Current limitations of low dose X-ray and electron crystallography of membrane proteins

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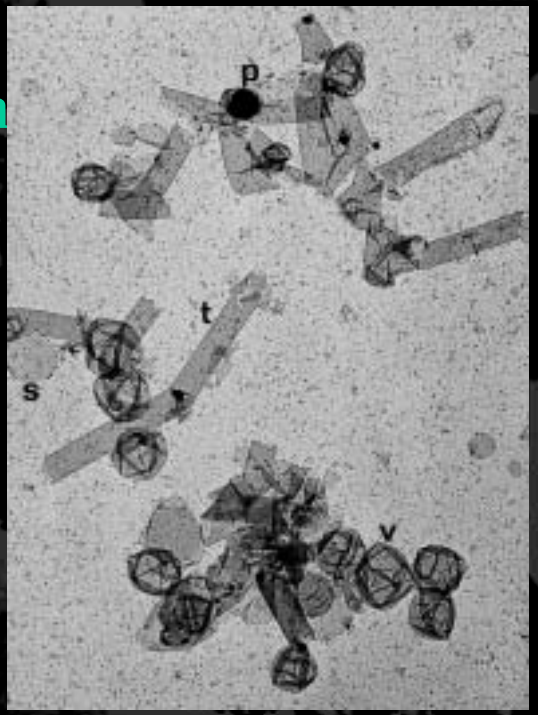


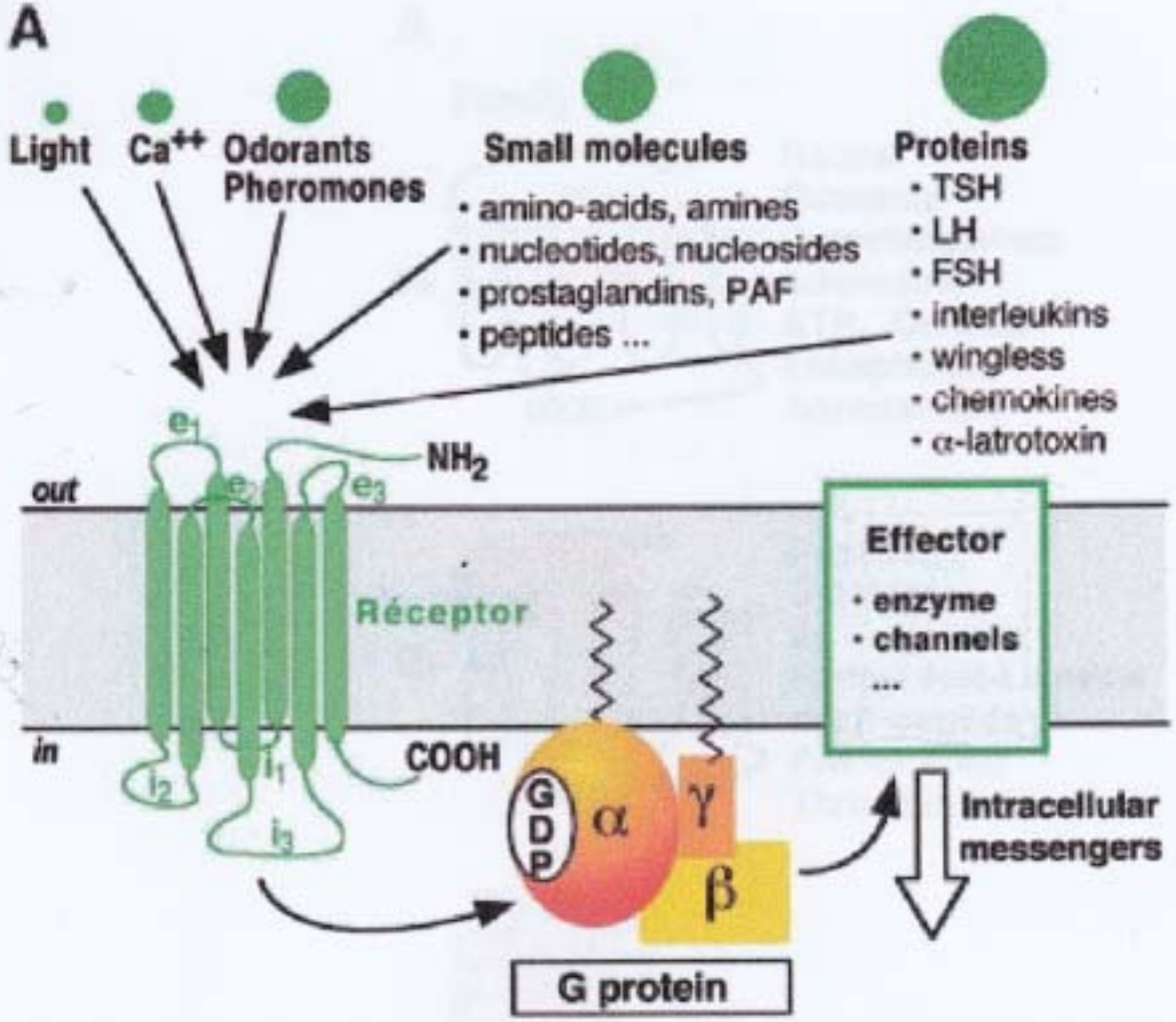
References for microdiffraction of protein crystals

Cusack, S., Belrhali, H., Bram, A., Burghammer, M., Perrakis, A. & Riekel, C. (1998)
Small is beautiful: protein micro-crystallography.
Nat Struct Biol 5 Suppl, 634-7.

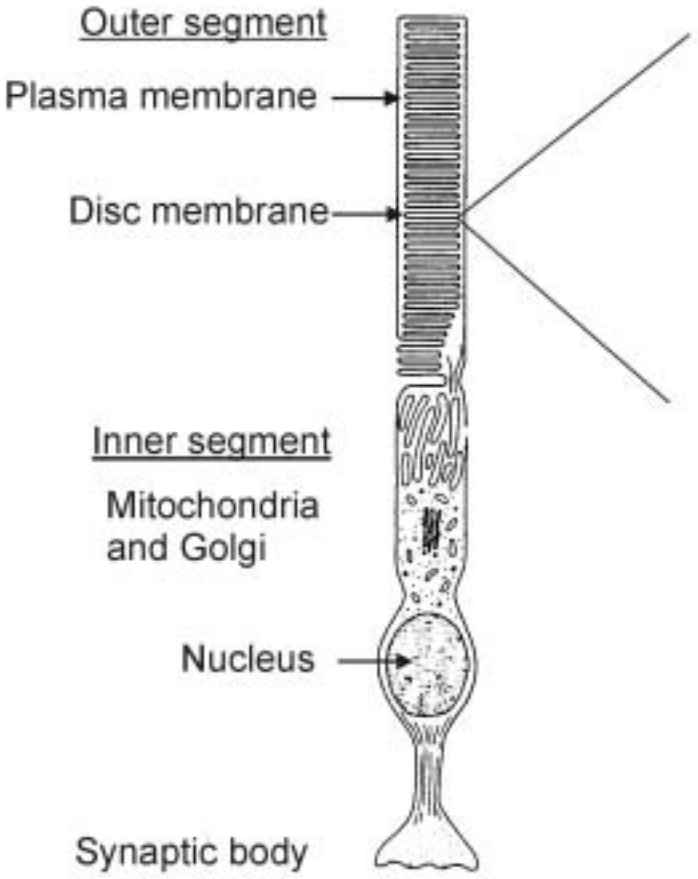
Perrakis, A., Cipriani, F., Castagna, J. C., Claustre, L., Burghammer, M., Riekel, C. & Cusack, S. (1999).
Protein microcrystals and the design of a microdiffractometer: current experience and plans at EMBL and ESRF/ID13.
Acta Crystallogr D Biol Crystallogr 55 (Pt 10), 1765-70.

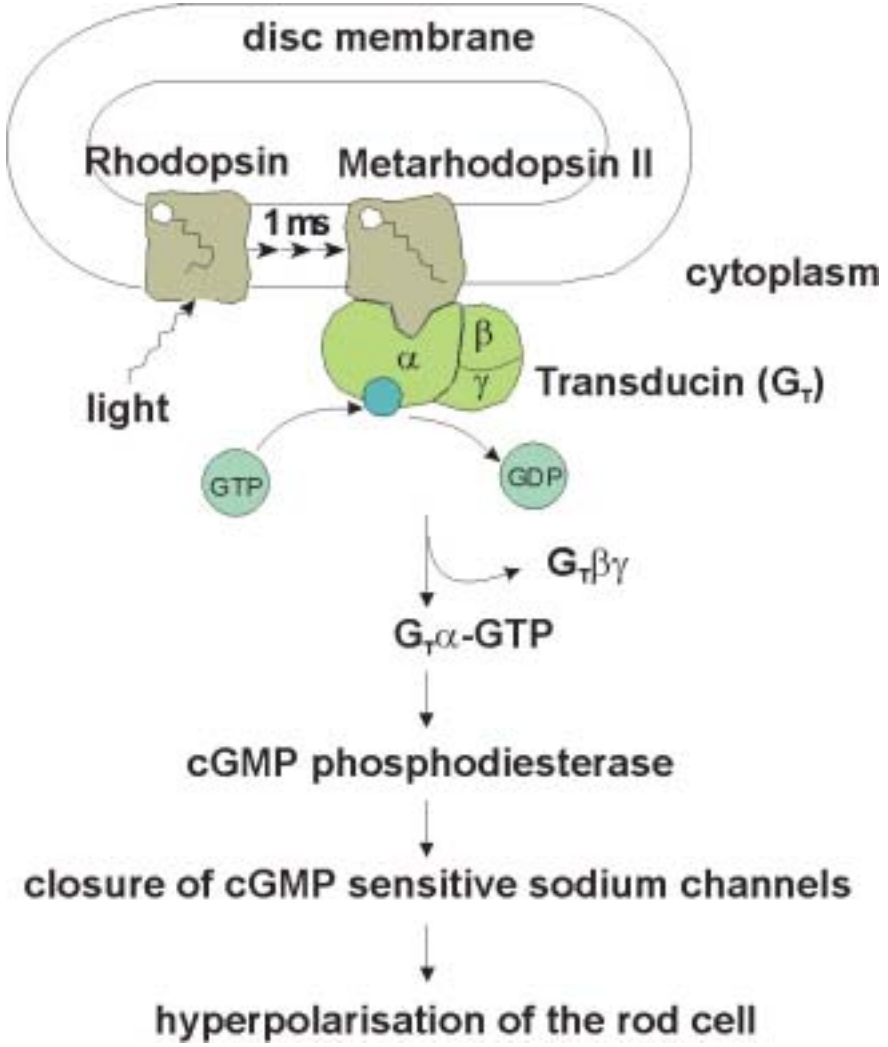
Riekel, C. (2004).
Recent developments in micro-diffraction on protein crystals.
J Synchrotron Radiat 11, 4-6.

J.Bockaert and J.P.Pin

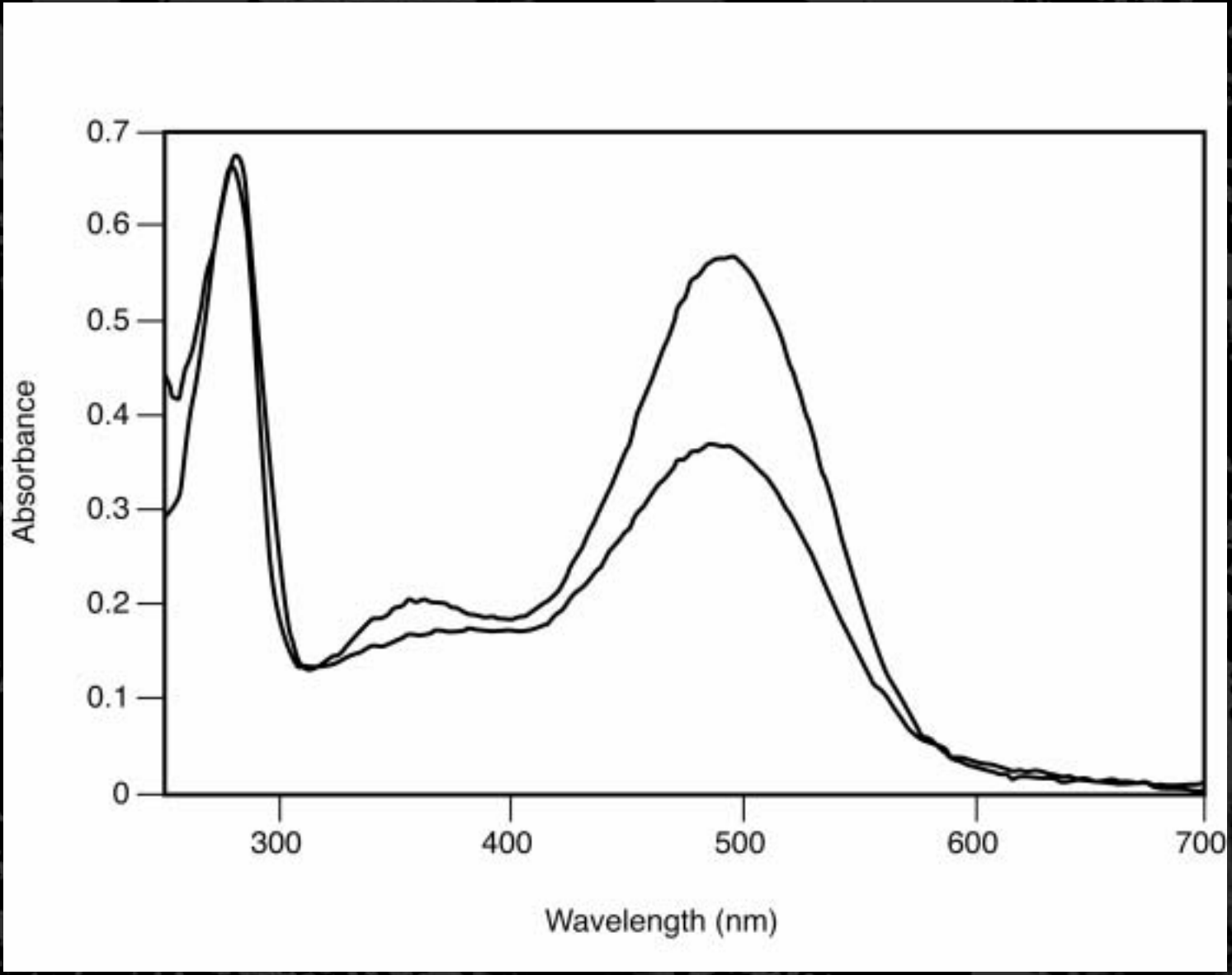


The visual signal transduction cascade

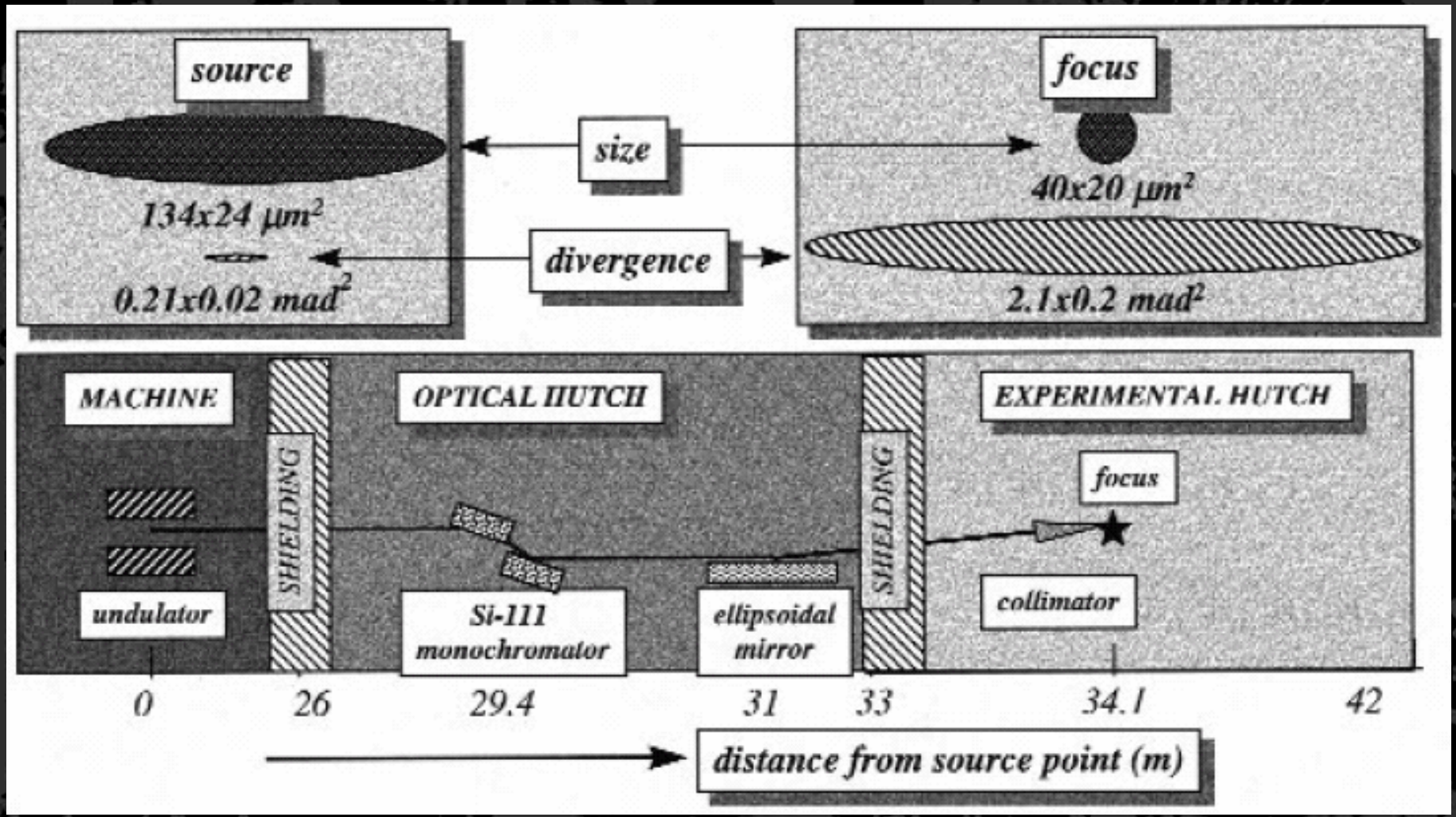




Polarised absorption spectra of P3(1) crystal

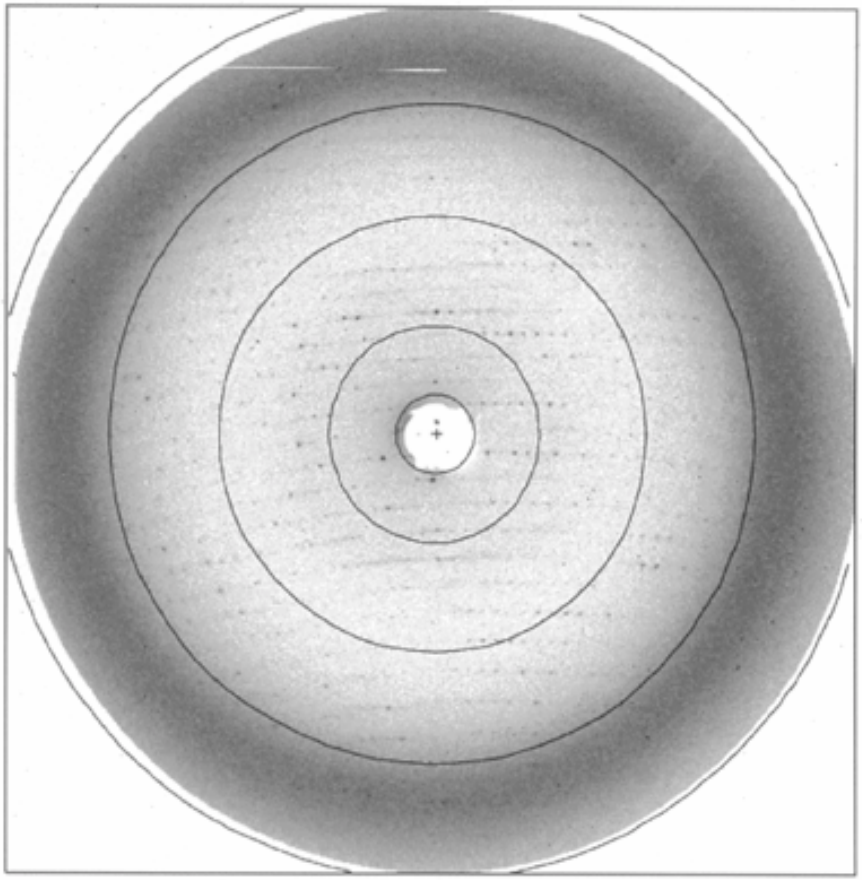


Microfocus beamline ID13 focusing optics

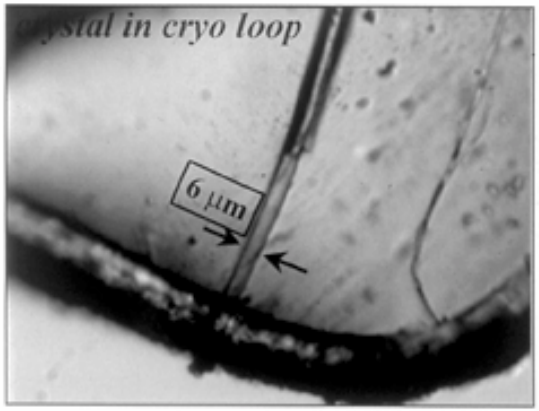


Prototype of microdiffractometer at ID13

Rhodopsin at ESRF Microfocus Beamline

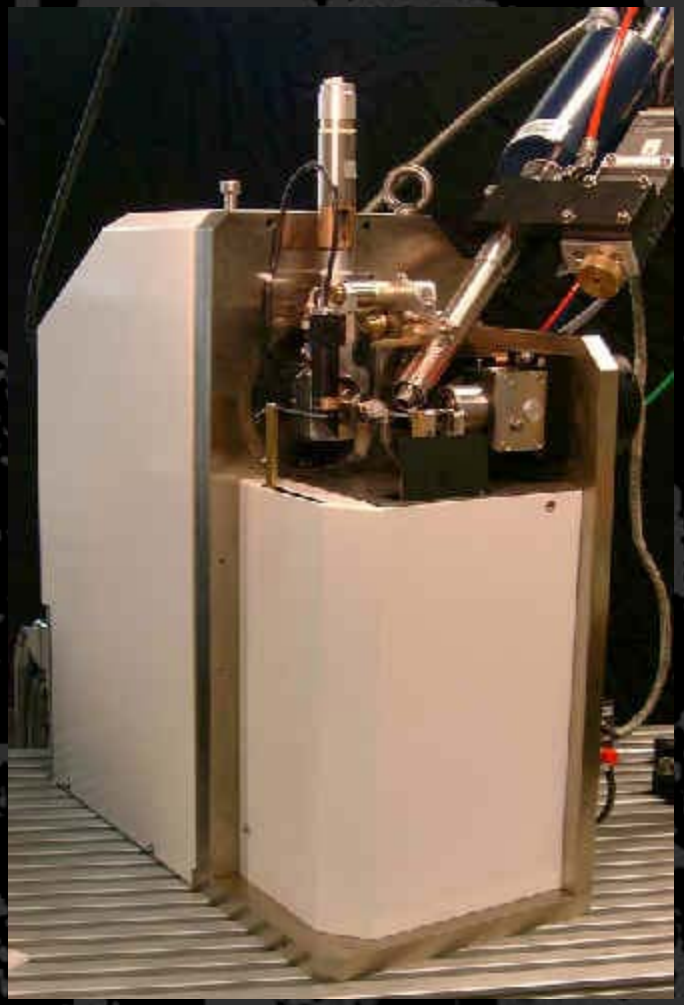


Bovine Rhodopsin at 110 K
λ=0.0782 nm
10 μm beam
1^0 rotation
20 sec exposure
MAR CCD
G. Schertler et al.,
LMB Cambridge UK



Crystal in cryo loop

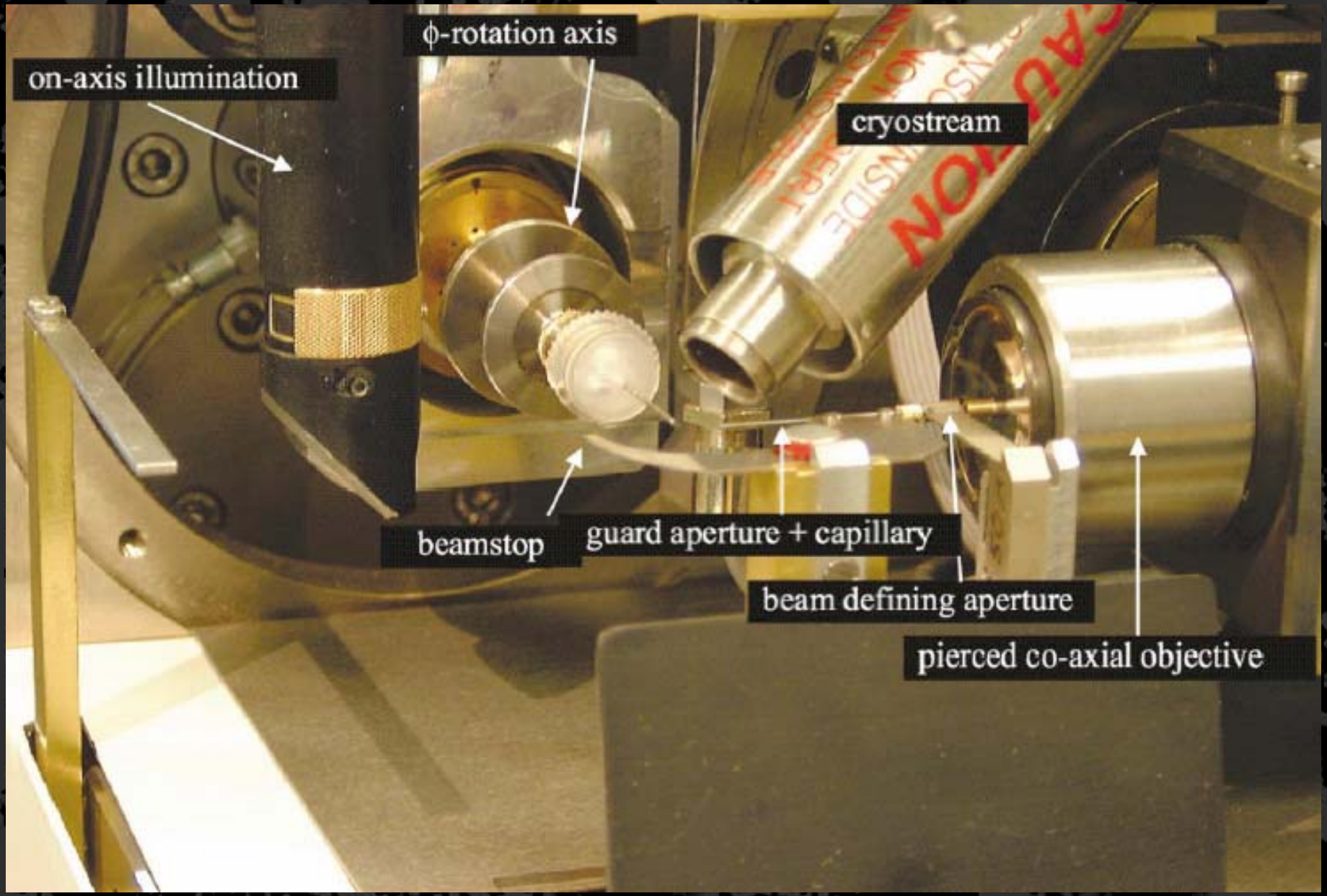
Microdiffractometer at the ESRF
ID13 Micro-focus beam line Grenobel



Manfred Burghammer
Chrisitan Riekel

EMBL and ESRF

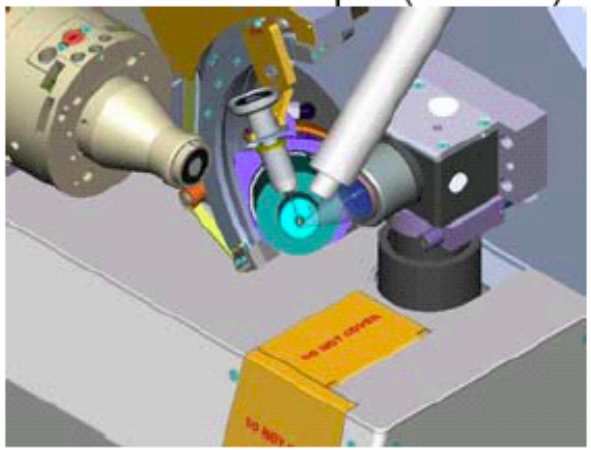
Sample environment of microgoniometer



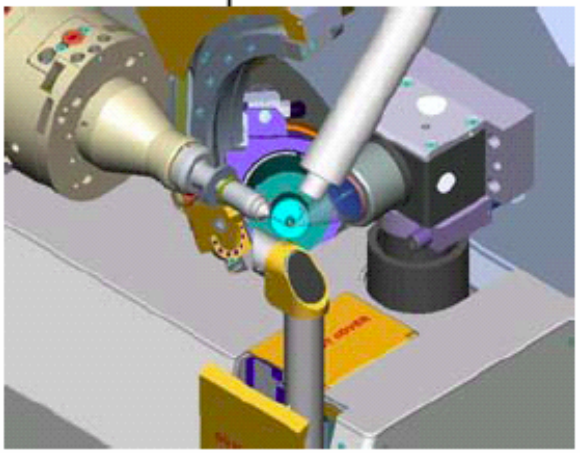
Fast and automatic sample loading

Examples of sample processing phases

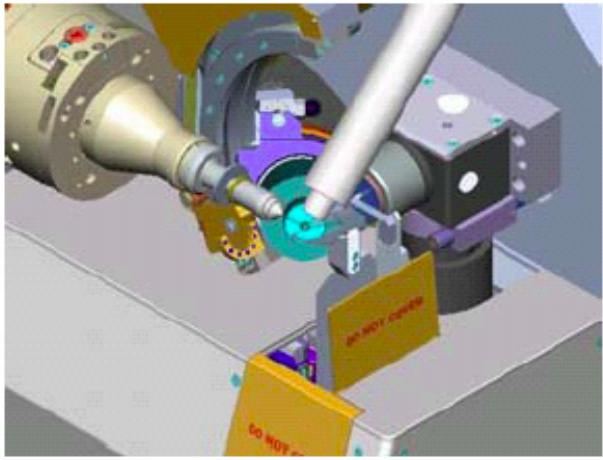
Load/unload Sample (arc use)



Centre Sample



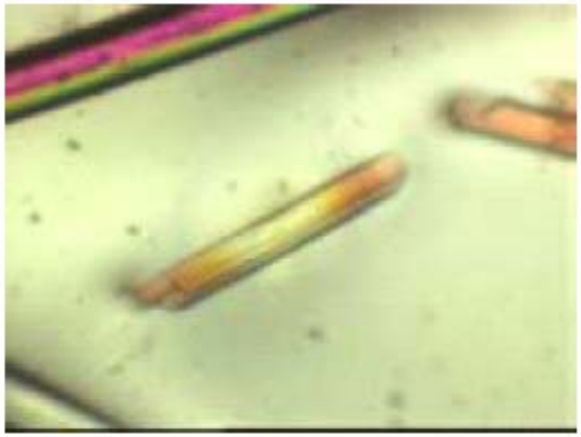
Data collection



MAATEL / EMBL

Video microscope ID13

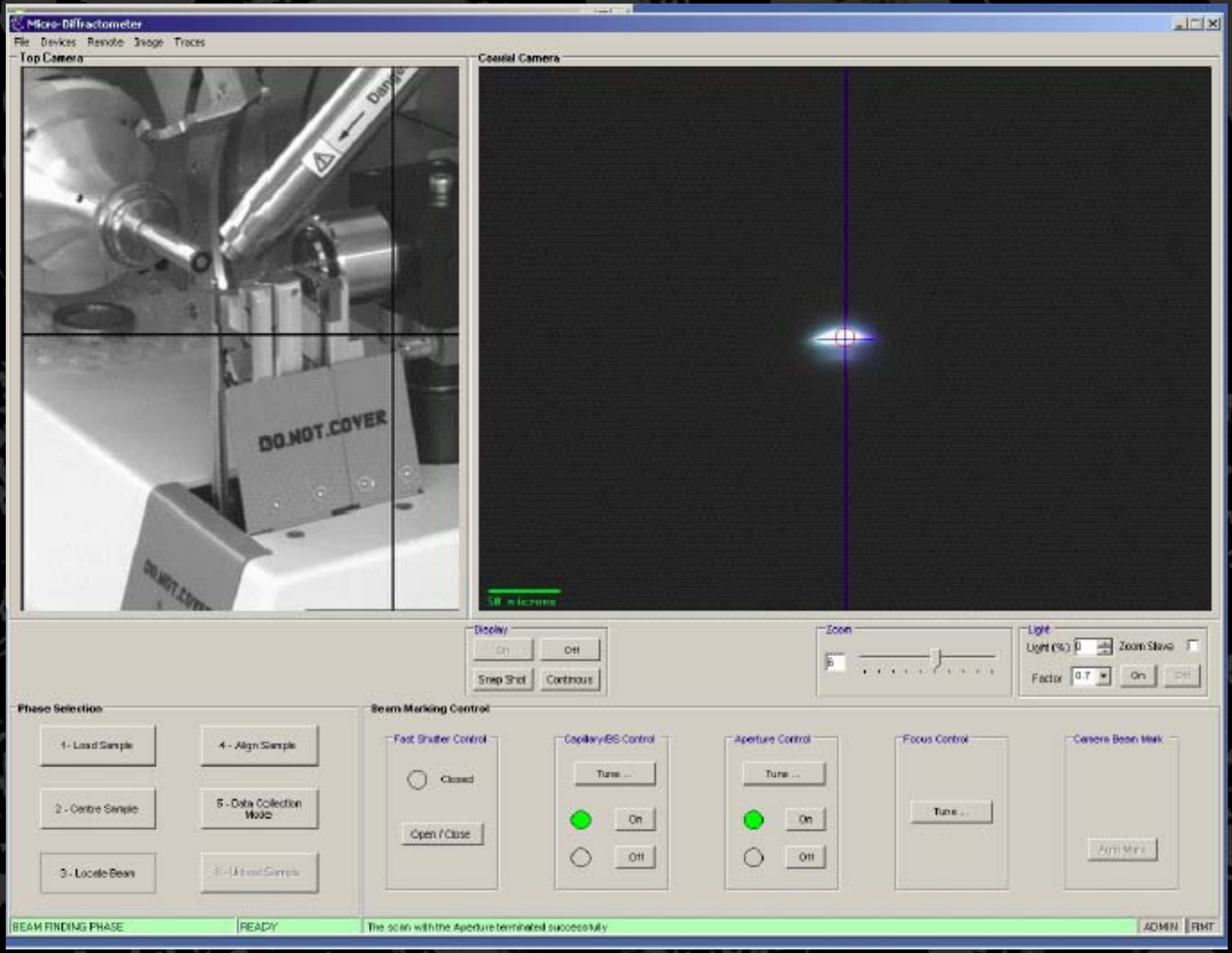
10 µm sample



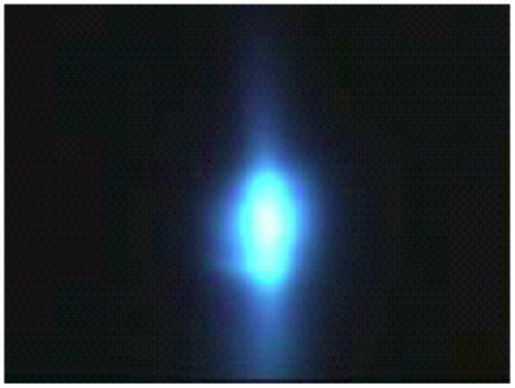
12µm needle (zoom 10)

30µm beam (zoom 6)

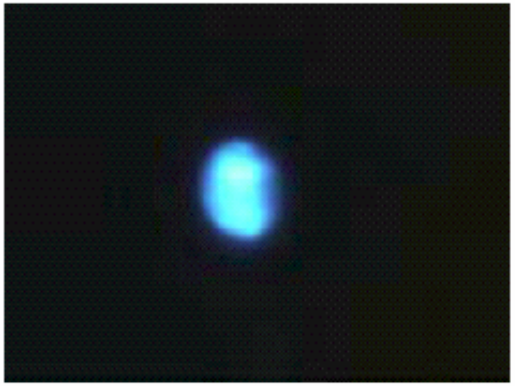
GUI in locate beam mode



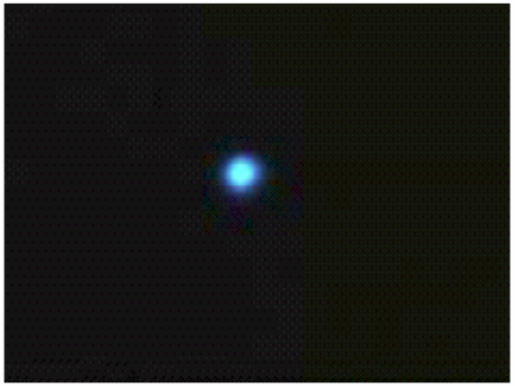
Beam shaping with guard tube and aperture



No shaping



100µm cleaning aperture



30µm definition aperture + 100µm cleaning aperture

Comercial Microdiffractometer from MAATEL

Crystallisation of Detergent solubilised Bovine Rhodopsin

Bovine retina

↓

Rod outer segments

↓

Solubilise in 1% LDAO

↓

ConA affinity chromatography

↓

Sephadex G50 in 0.2% C8E4

↓

Mono Q anion exchange in C8E4

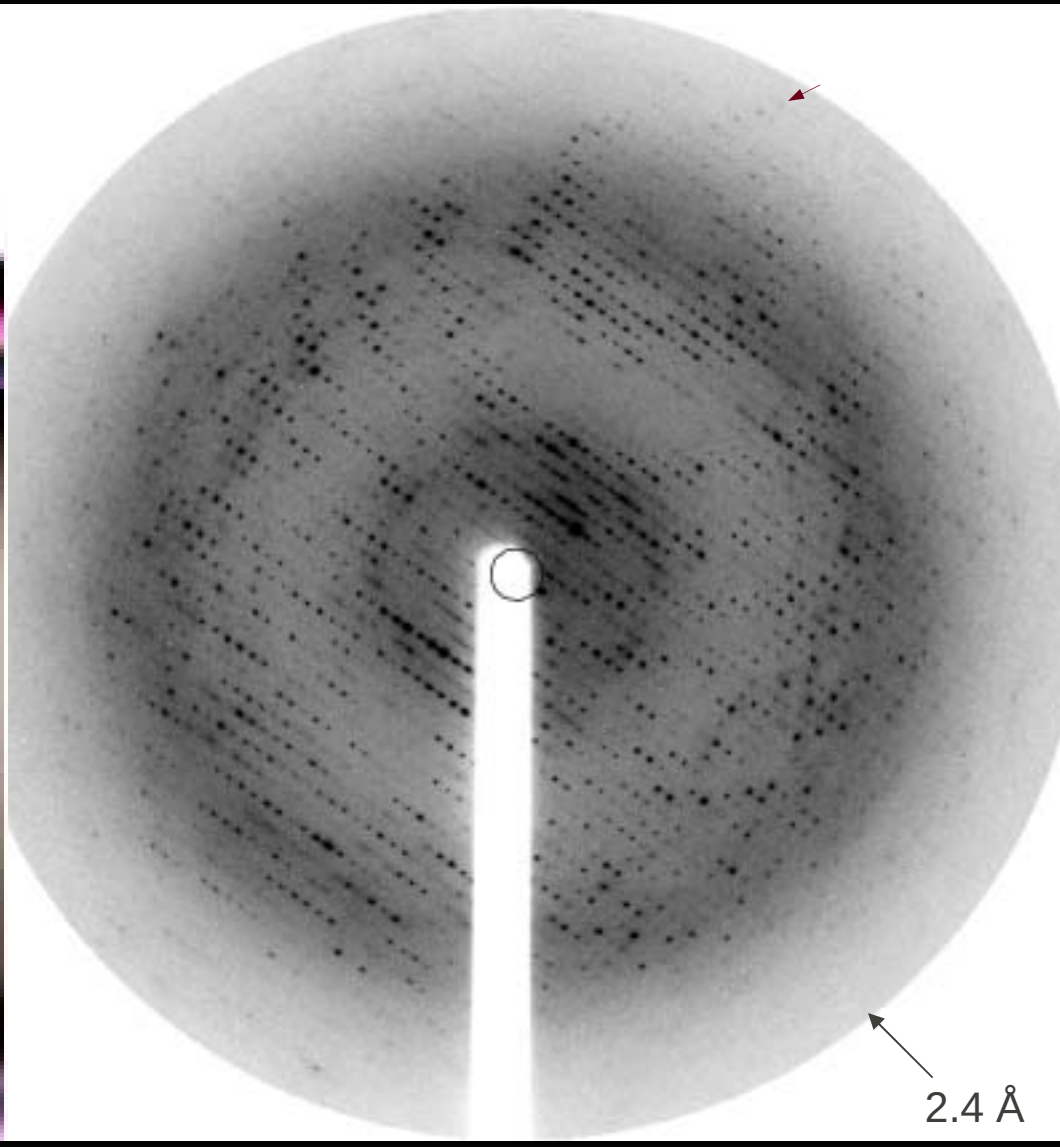
Sitting Drop Vapour Diffusion

Single fractions from Mono Q
15 mg/ml Protein
0.2% C8E4
0.05% LDAO
0.8M Li_2SO_4
1.6% PEG8000
20% Glycerol

Freeze in liquid Ethane or Nitrogen

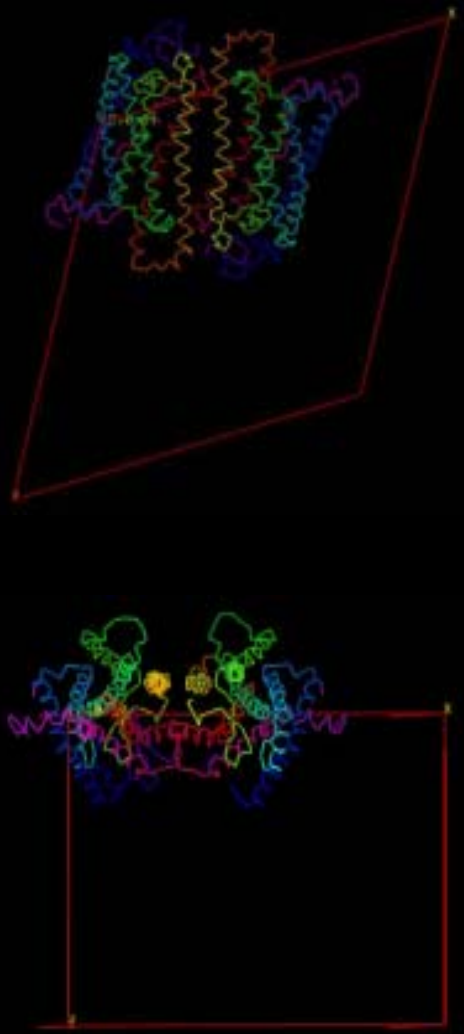
Everything needs to be done in a dark lab!

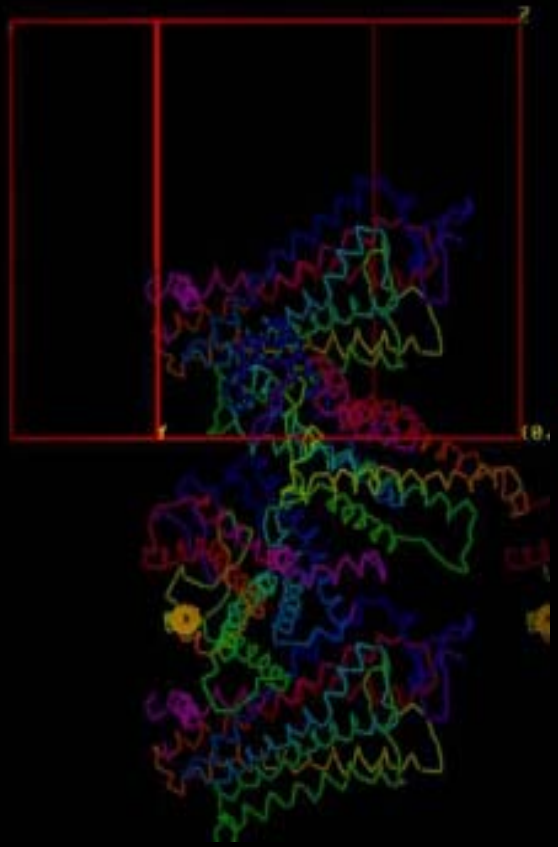

50 μ


2.4 Å

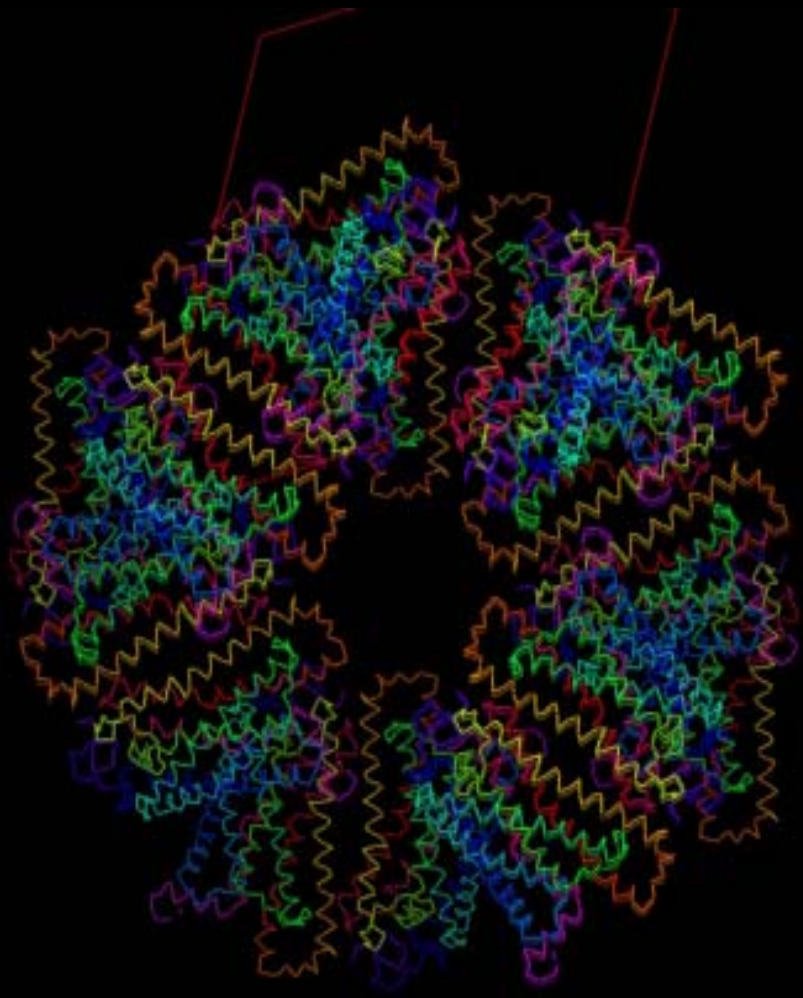
Packing in the $P3_1$ Lattice

Anti-parallel dimer



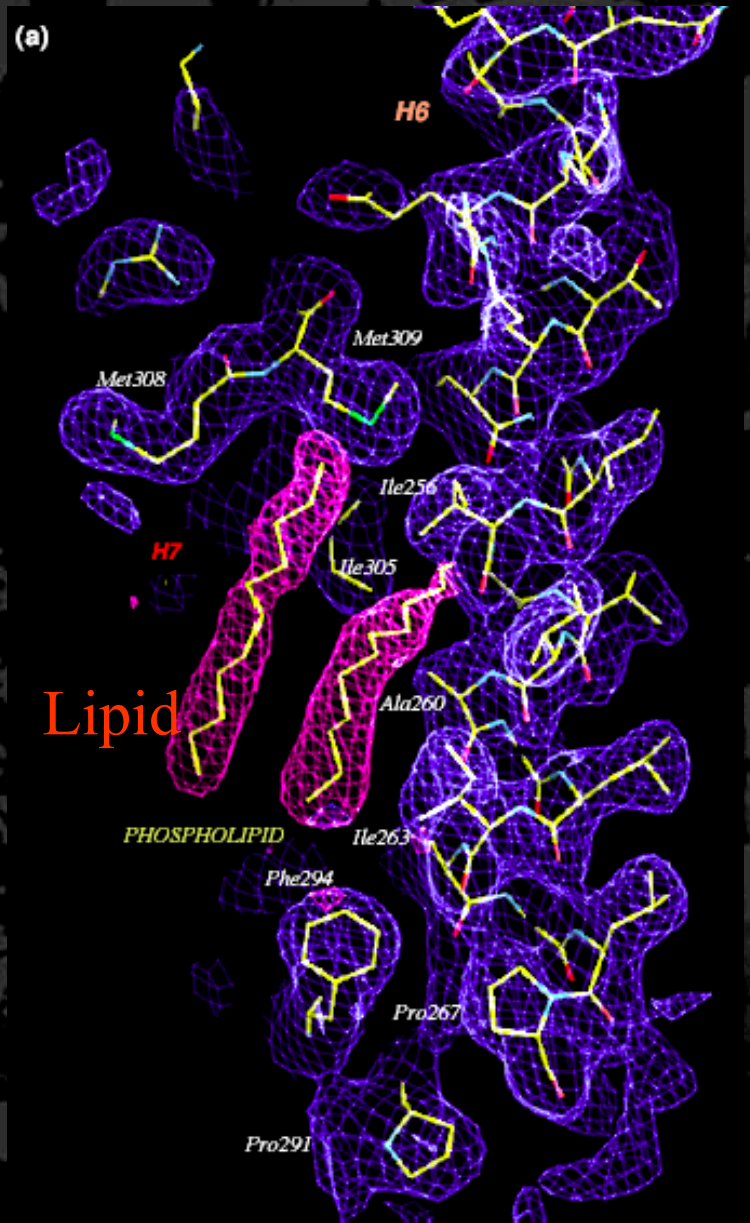


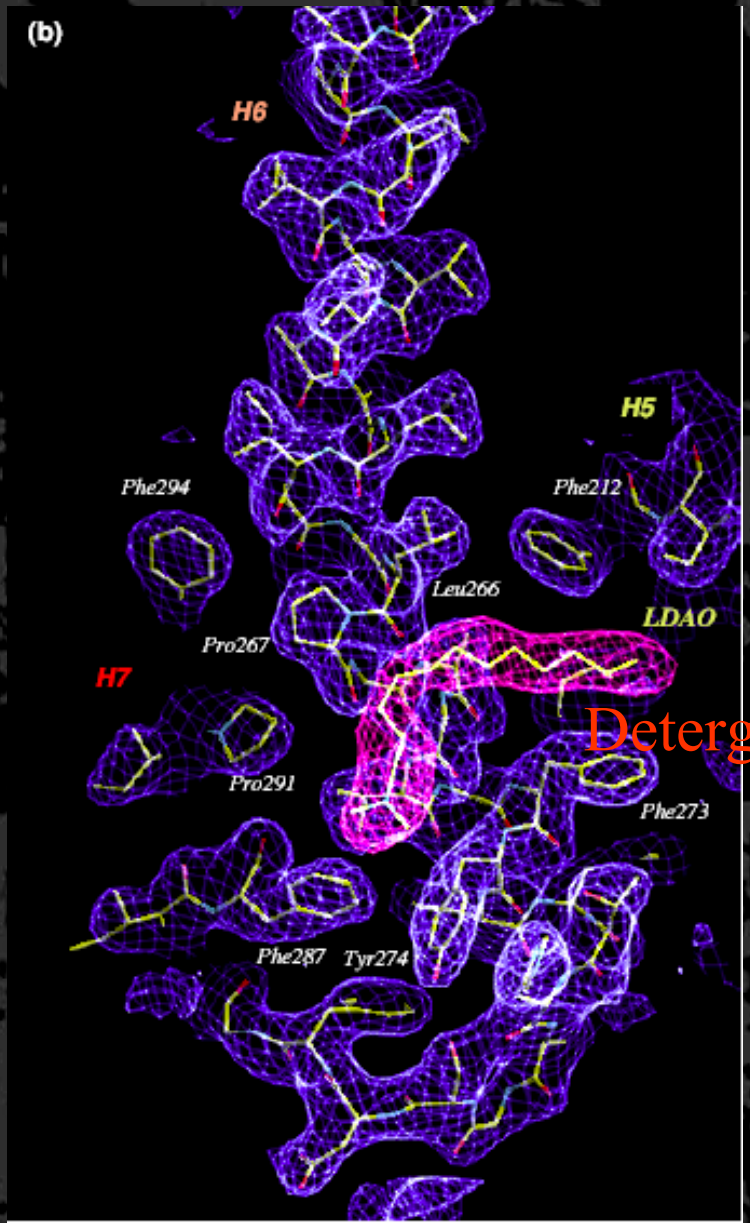
Stacking along the 3_1-axis

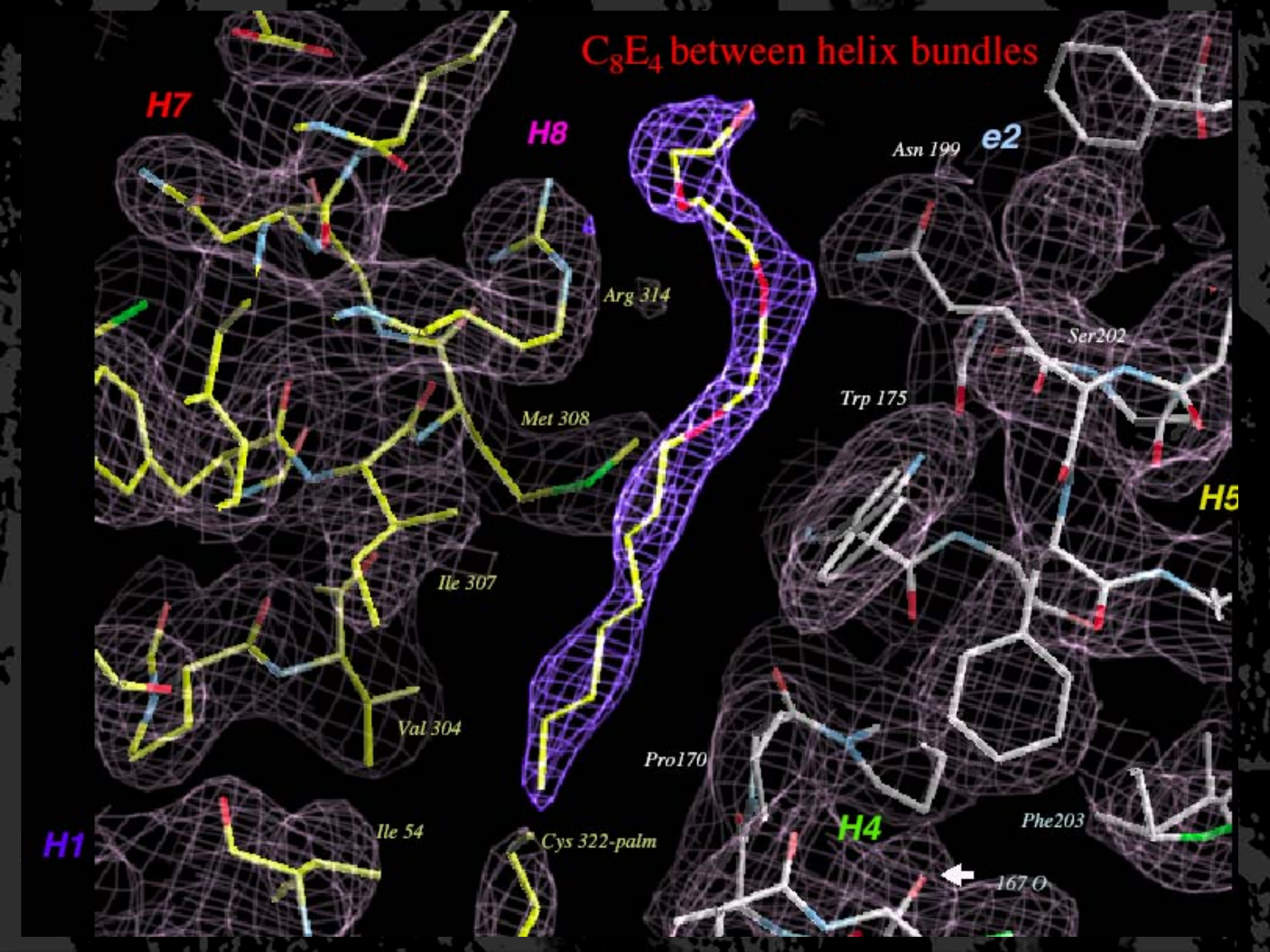


Cytoplasmic loops
in solvent channel

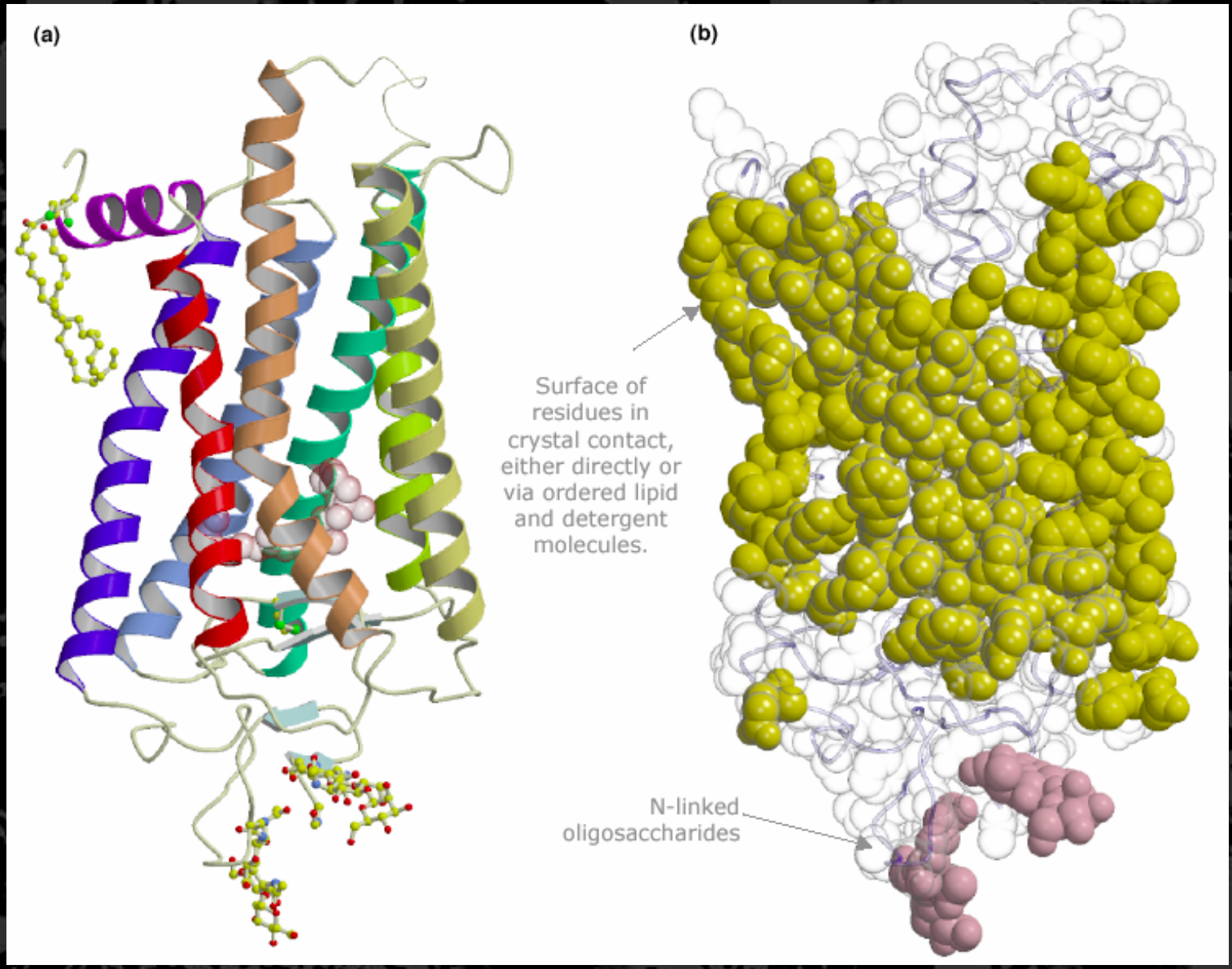
Detergents and lipids bound to rhodopsin in P3(1) crystal






C_8E_4 between helix bundles
H7
H8
e2
Asn 199
Arg 314
Ser202
Trp 175
Met 308
H5
Ile 307
Val 304
Pro170
H1
Ile 54
Cys 322-palm
H4
Phe203
167 O

Hydrophobic intaractions in P3(1) rhodopsin crystal



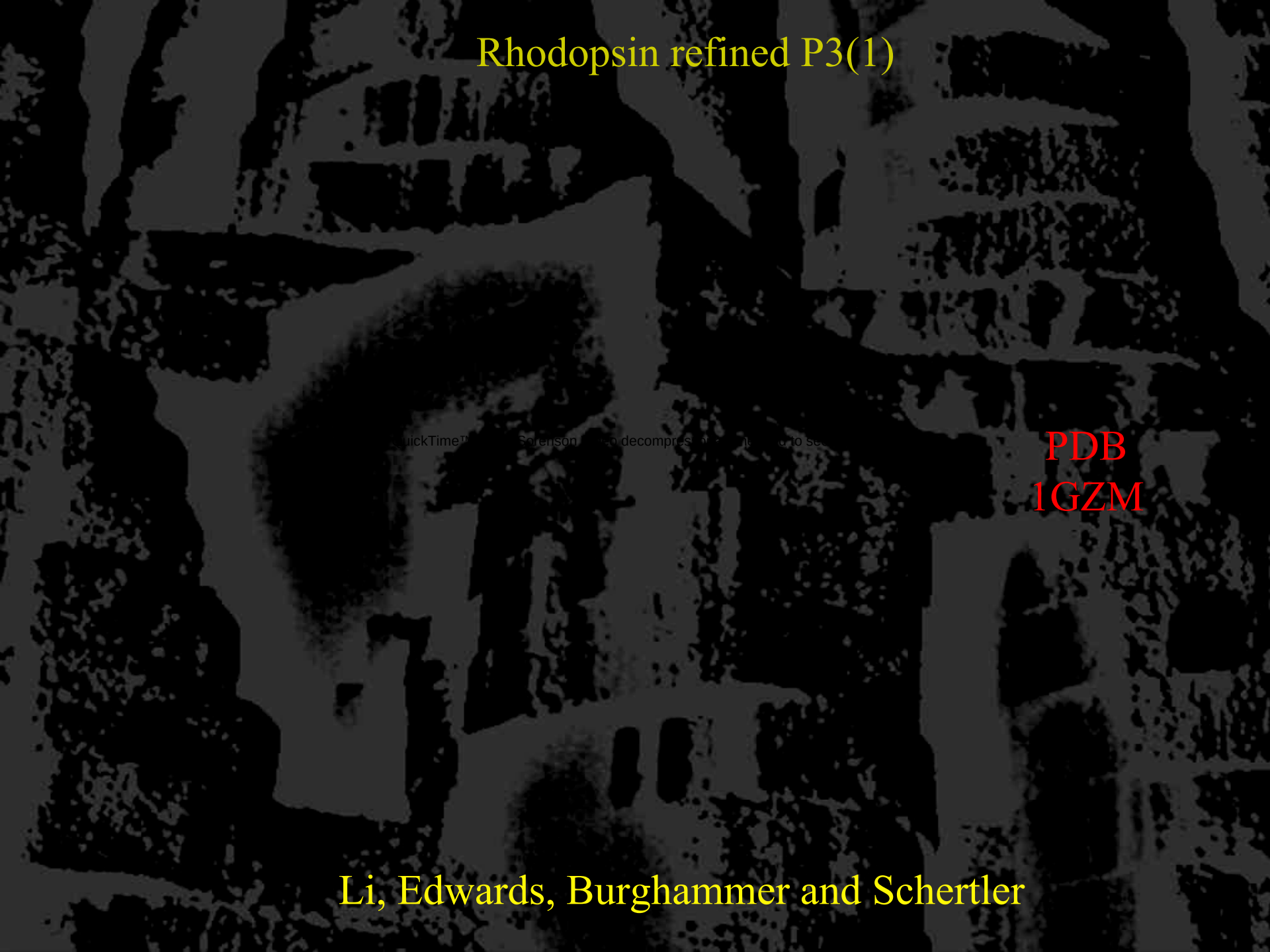
Rhodopsin P3(1) data collection

Data collection	Native-2[b]	EMTS-1	EMTS-2	Native-3
X-ray Source	ESRF ID13	ESRF ID13	ESRF ID14-4	ESRF ID13
Wavelength (Å)	0.782	1.005	1.0065	0.782
Unit cell ***a, c*** (Å)	104.2, 77.1	113.9, 78.4	109.3, 77.6	103.8, 76.6
No. of crystals	2	1	1	4
Mosaicity (°)	1.0	1.1	1.1	0.75
Twin fraction	0	0	0.31	0
Resolution (Å)	3.2 (3.37-3.20)	3.6 (3.71-3.60)	3.4 (3.45-3.40)	2.65 (2.79-2.65)
R_{merge}[c]	0.127(0322)	0.169(0.464)	0.139(0.426)	0.119(0.434)
I/σ	7.5 (2.3)	7.6 (2.2)	17.2 (4.7)	11.0 (1.4)
Unique reflections	15087	11481	13320	26026 (2295)
Completeness (%)	0.982 (0.894)	0.868 (0.180)	0.834 (0.172)	0.970 (0.861)
Multiplicity	3.1 (1.6)	5.3 (2.9)	11.3 (9.5)	4.4 (1.6)
Wilson B ($Å^2$)	84.6	59.7	54.2	58.2

Rhodopsin P3(1) refinement statistics

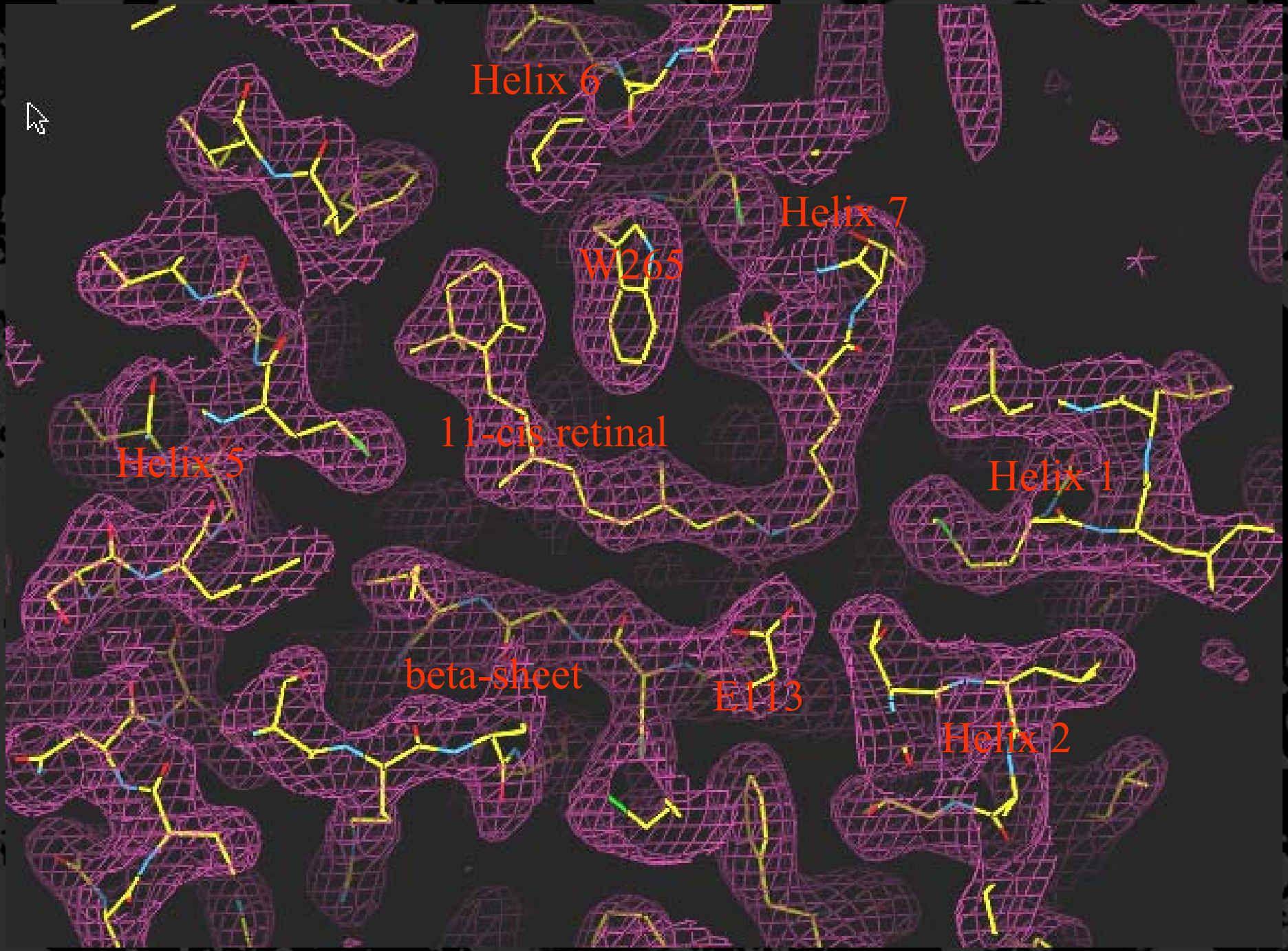
Reflections in working set	24704 (2165)	Protein chains	2
Reflections in test set	1322 (130)	Protein residues	652
Resolution range (Å)	46-2.65 (2.74-2.65)	Palmitoyl chains	4
R_{cryst}[d]	0.202 (0.312)	N-linked carbohydrate chains	4
R_{free}[e]	0.235 (0.315)	Carbohydrate residues	12
Luzzati coordinate error (5.0-2.65 Å)	0.31 Å	LDAO	2
SigmaA coordinate error (5.0-2.65 Å)	0.42 Å	C_8E_4	12
Rms-deviation from ideal geometry:		Phospholipid	2
bond lengths (Å)	0.008	Water	40
bond angles (°)	1.293	Ions	2
dihedral angles (°)	18.7		
improper rotations (°)	0.876		
Ramachandran plot: % Residues in			
most favoured regions	90.6		
additional allowed regions	7.1		
generously allowed regions	2.4		
disallowed regions	0		
Average B-factor (Å^2)	56.0		
B rmsd for bonded main chain atoms (Å^2)	1.501		
B rmsd for bonded side chain atoms (Å^2)	1.996		
B rmsd for angle main chain atoms (Å^2)	2.624		
B rmsd for angle side chain atoms (Å^2)	3.134		

Rhodopsin refined P3(1)

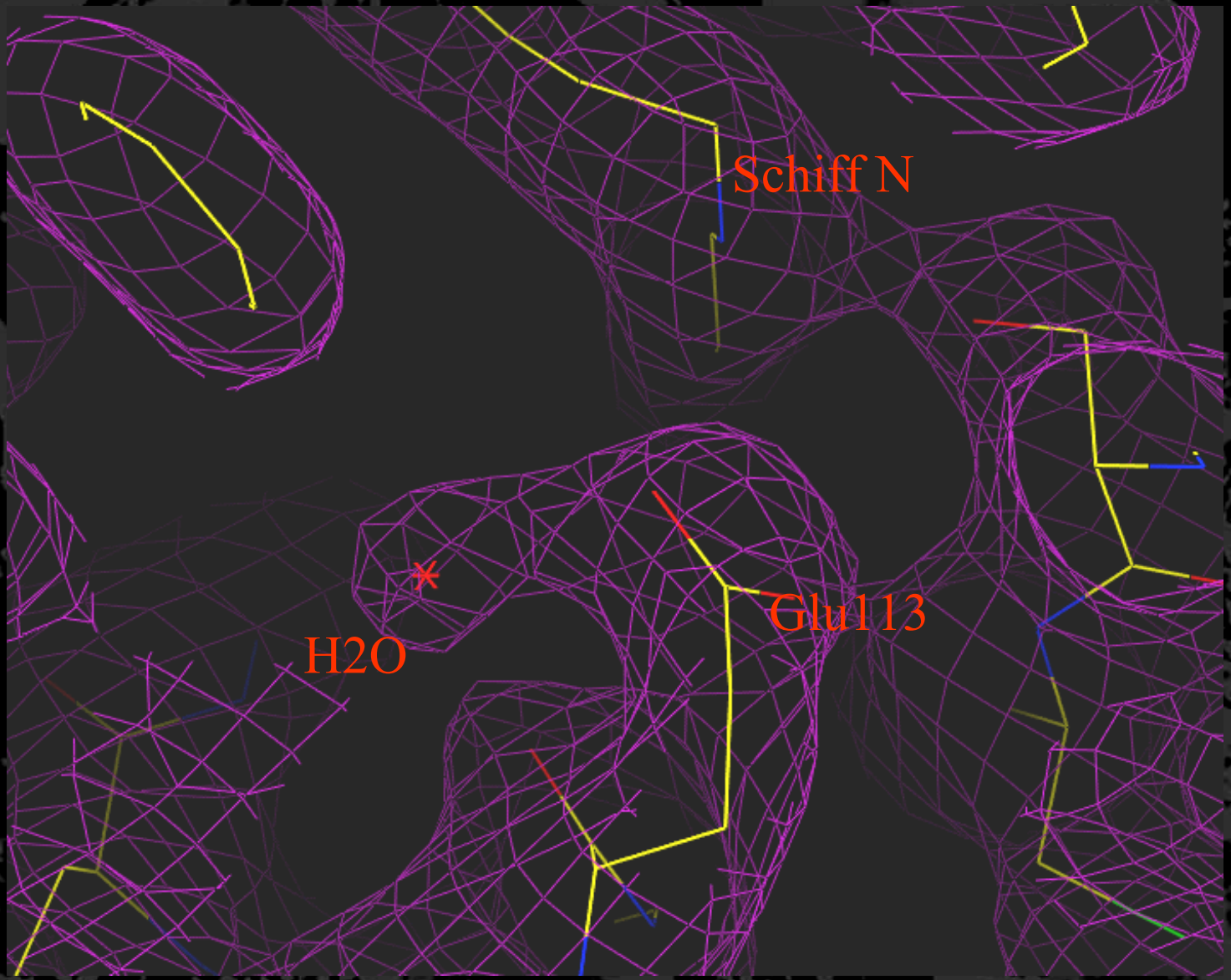


PDB
1GZM

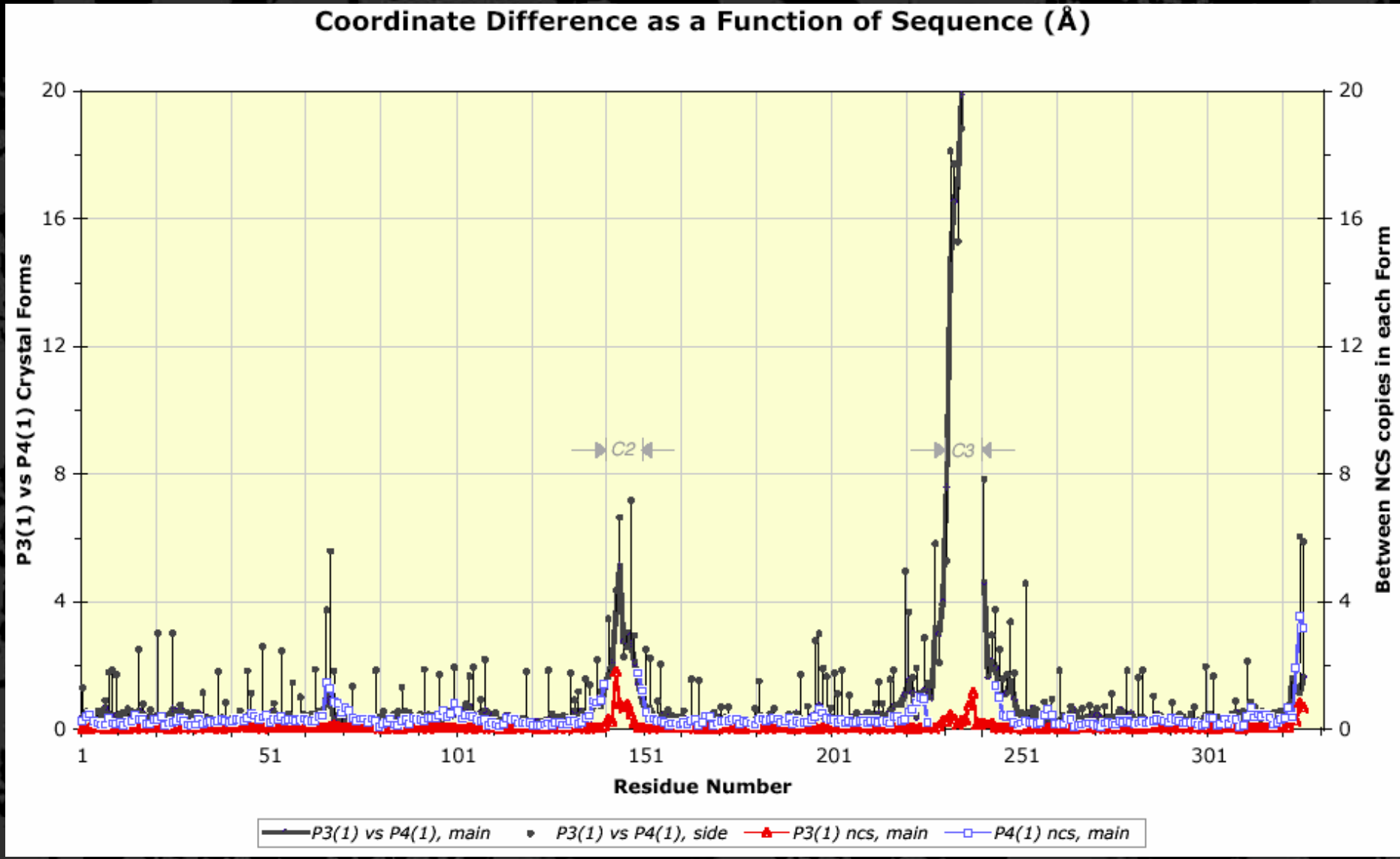
Li, Edwards, Burghammer and Schertler


Helix 6
Helix 7
W265
11-cis retinal
Helix 5
Helix 1
beta-sheet
E113
Helix 2

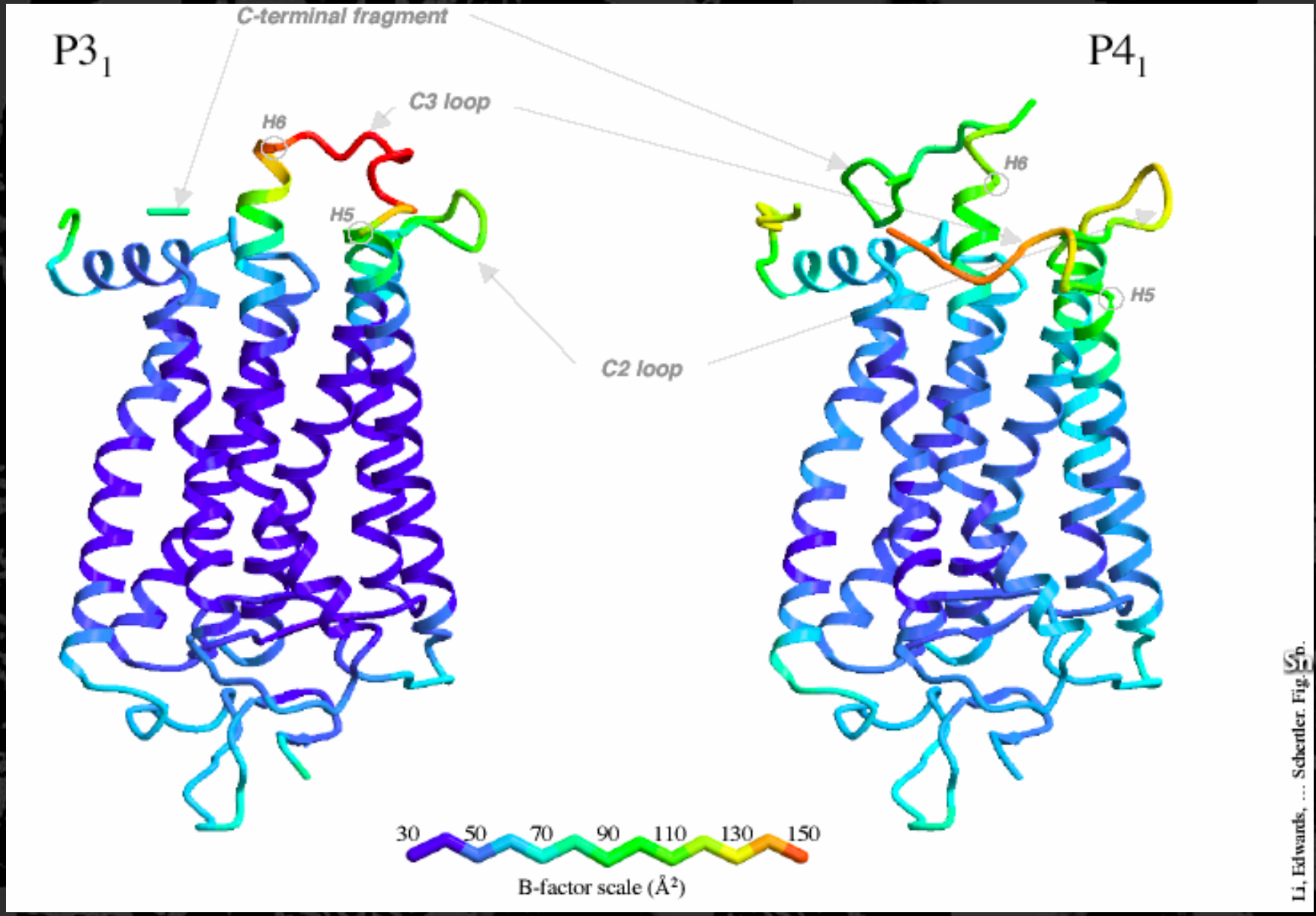
H2O close to Glu113 counterion



Comparing P4(1) and P3(1) coordinates

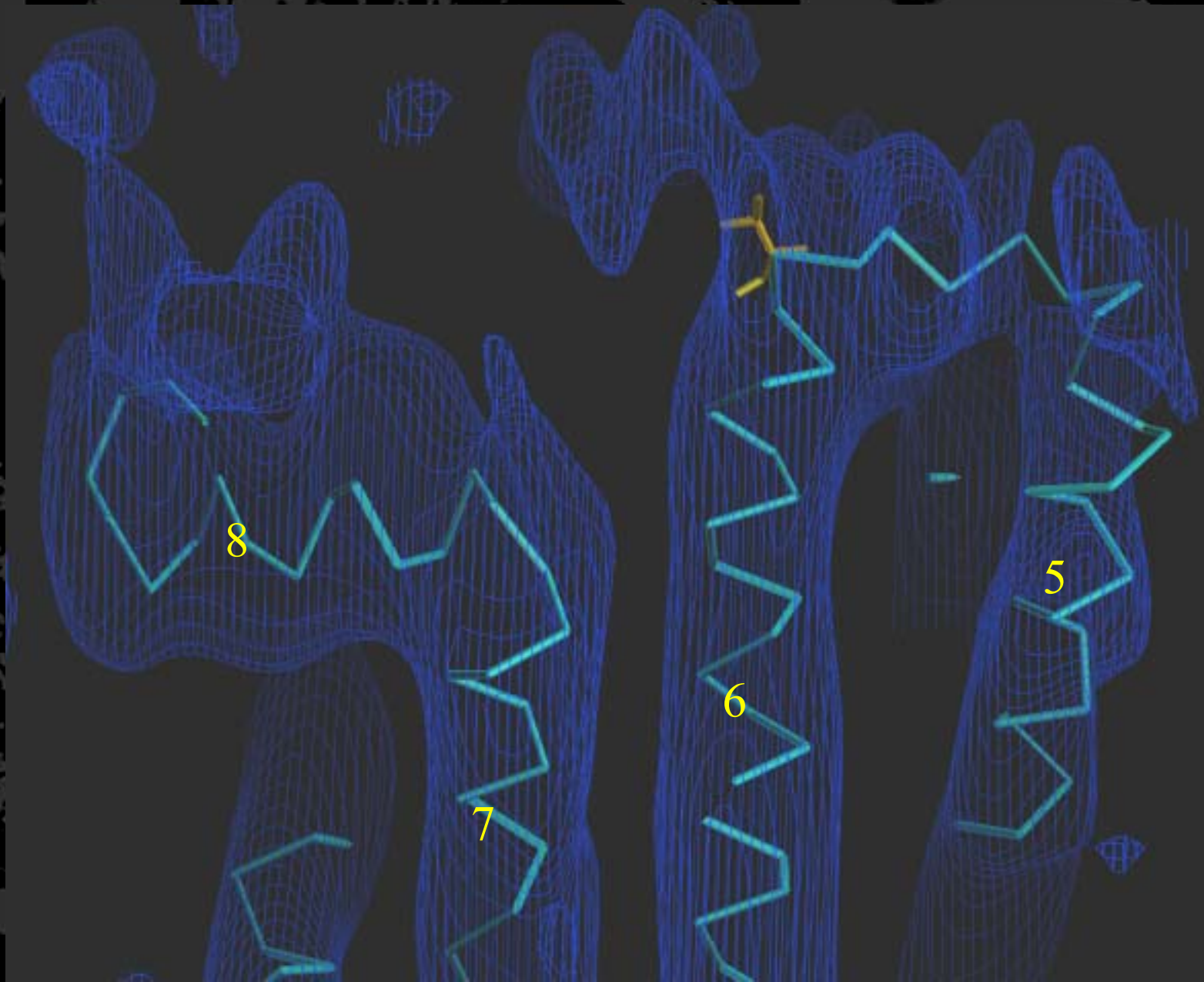


Comparison of P3(1) structure with P4(1) structure

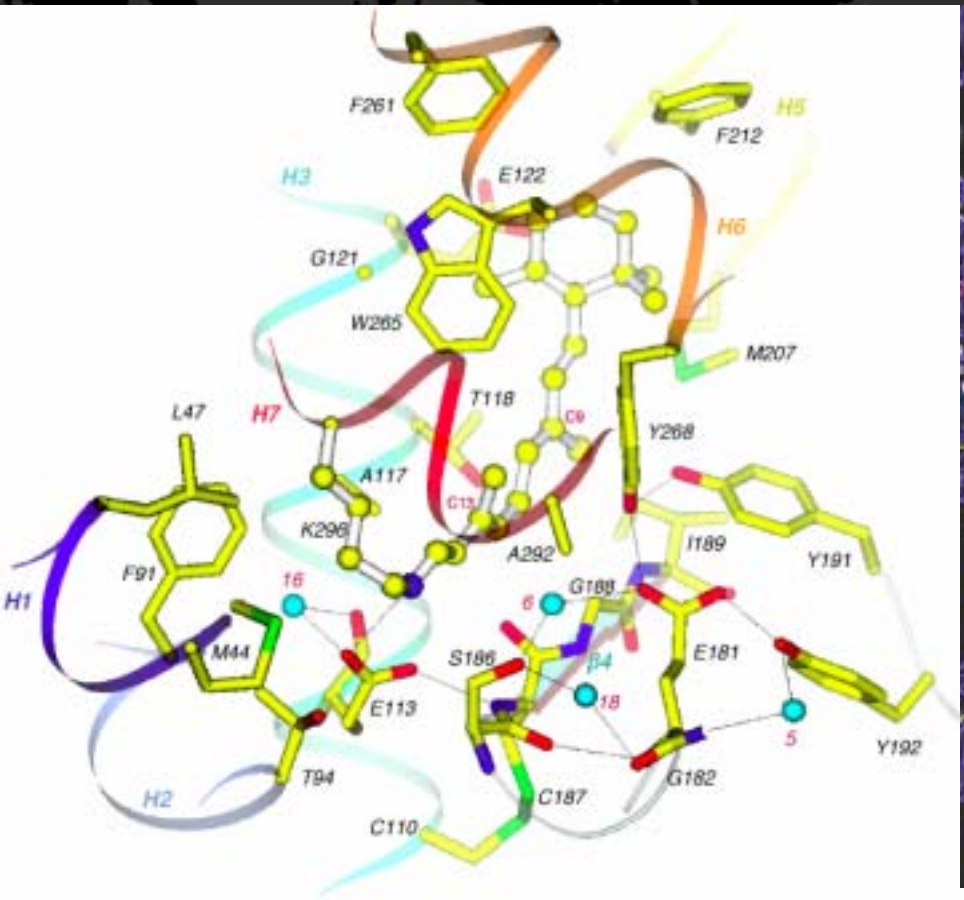


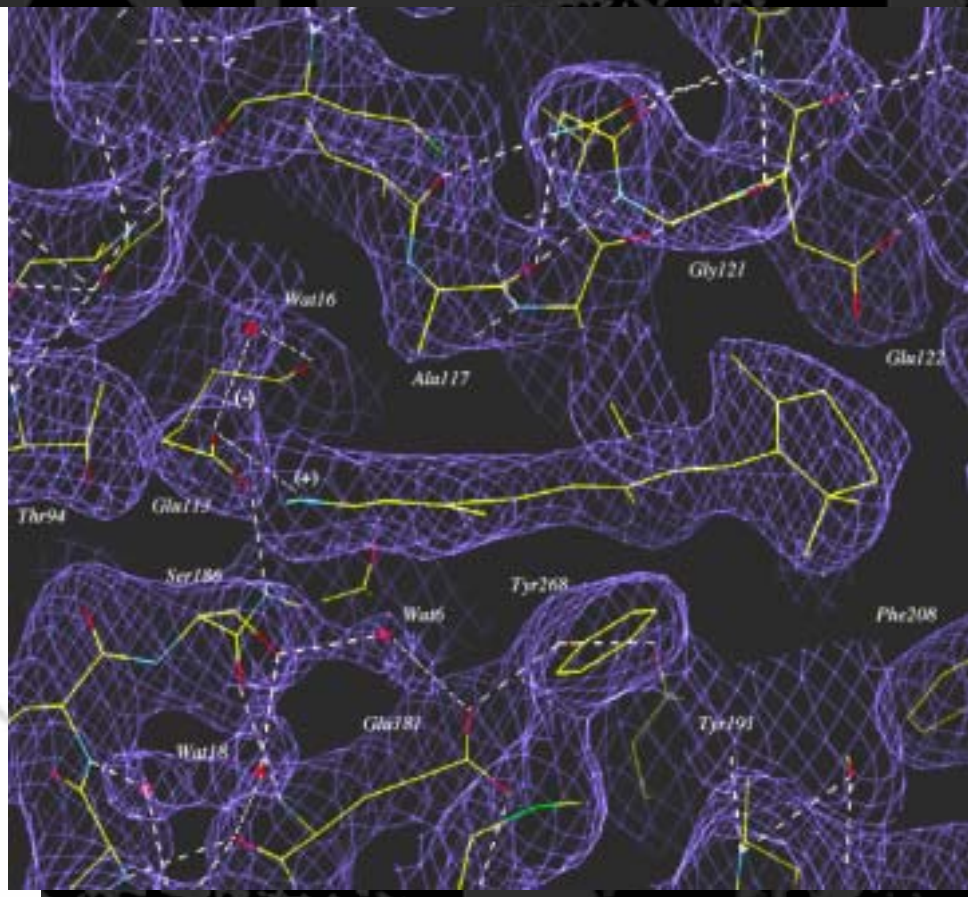
Li, Edwards, ... Schertler. Fig. 5b.

Helix 8 and 5 6 loop seen by cryo EM

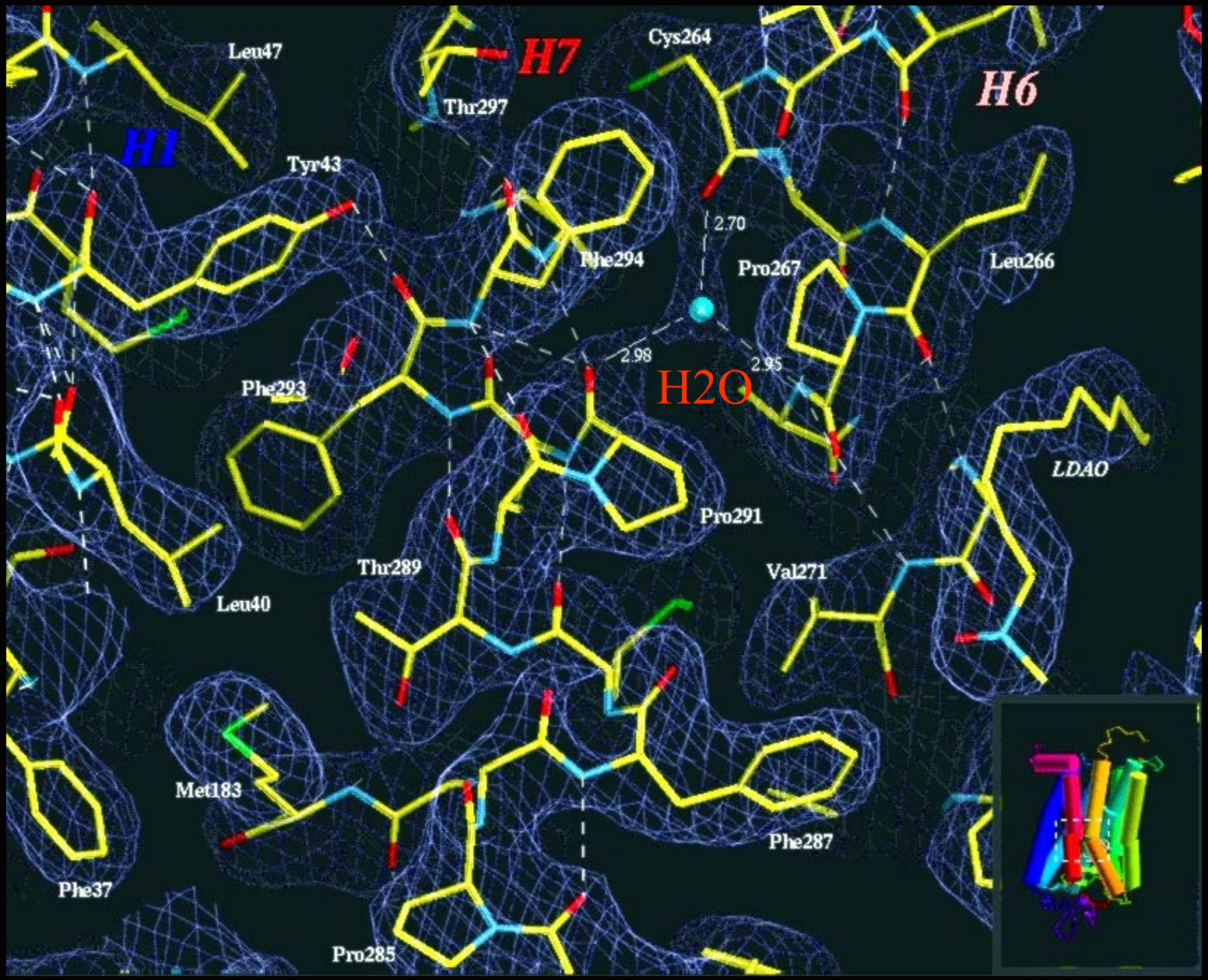


Rhodopsin lignad binding site: complex counter-ion

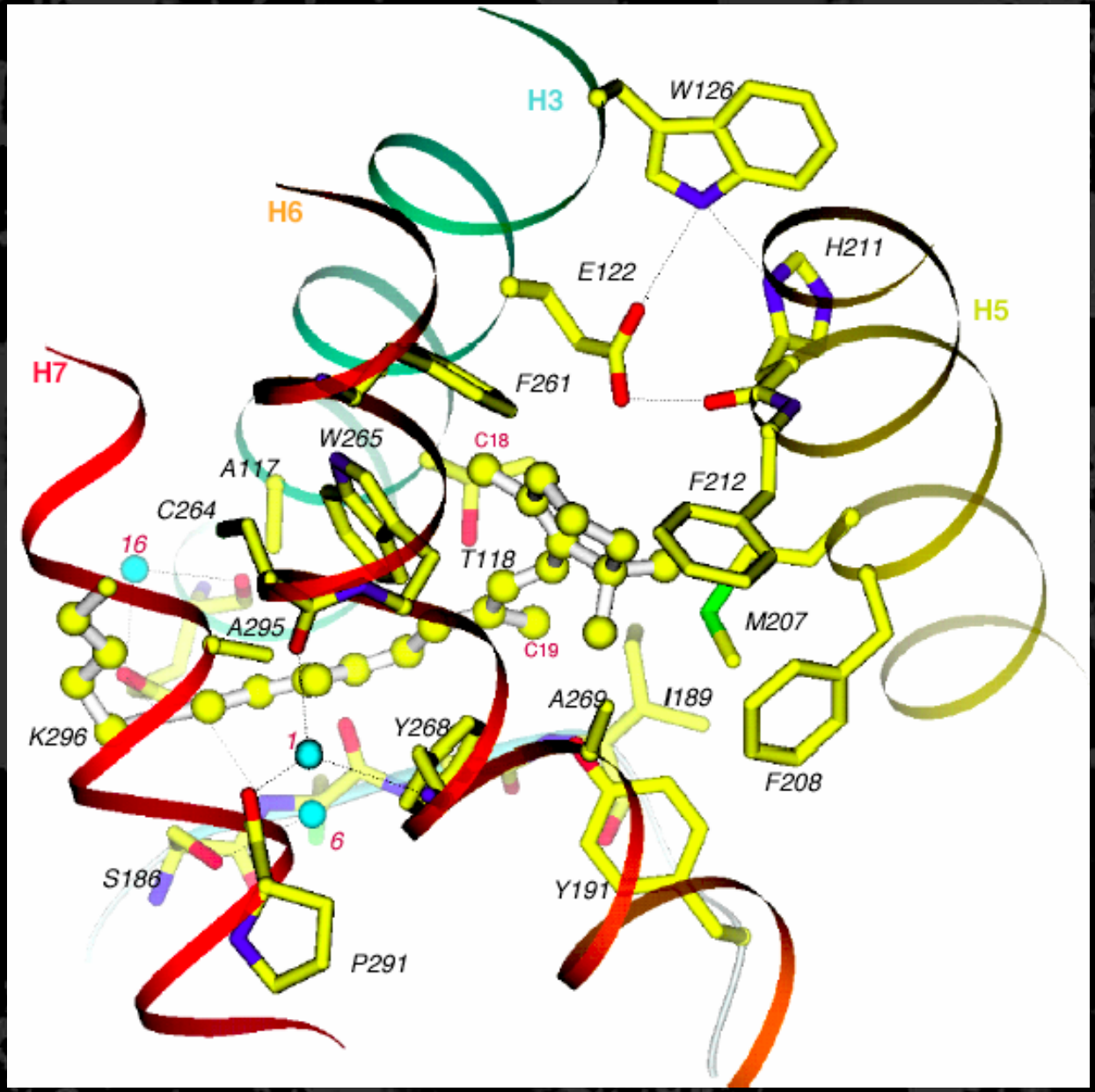




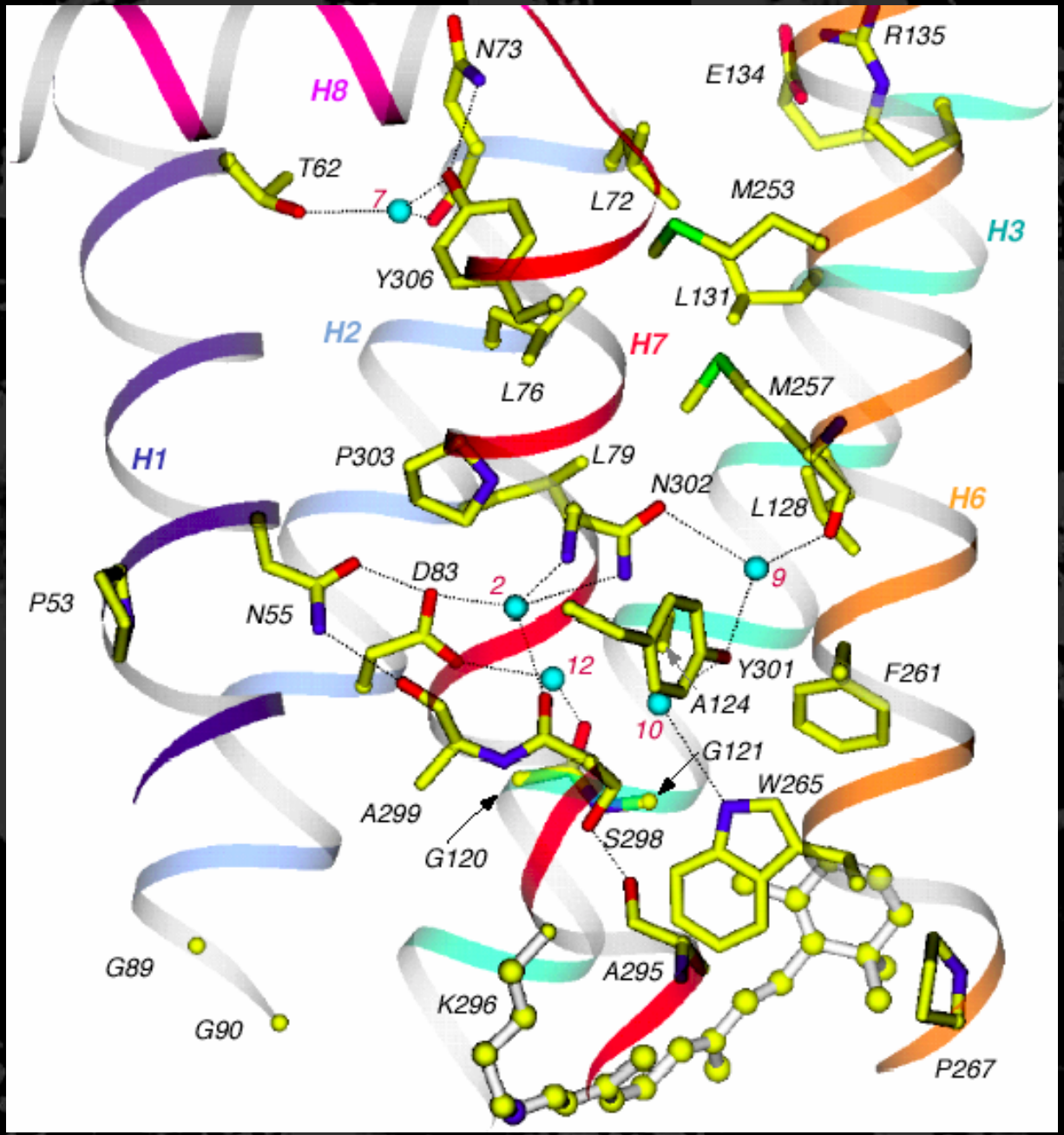
Water Between Helix 6 and Helix 7



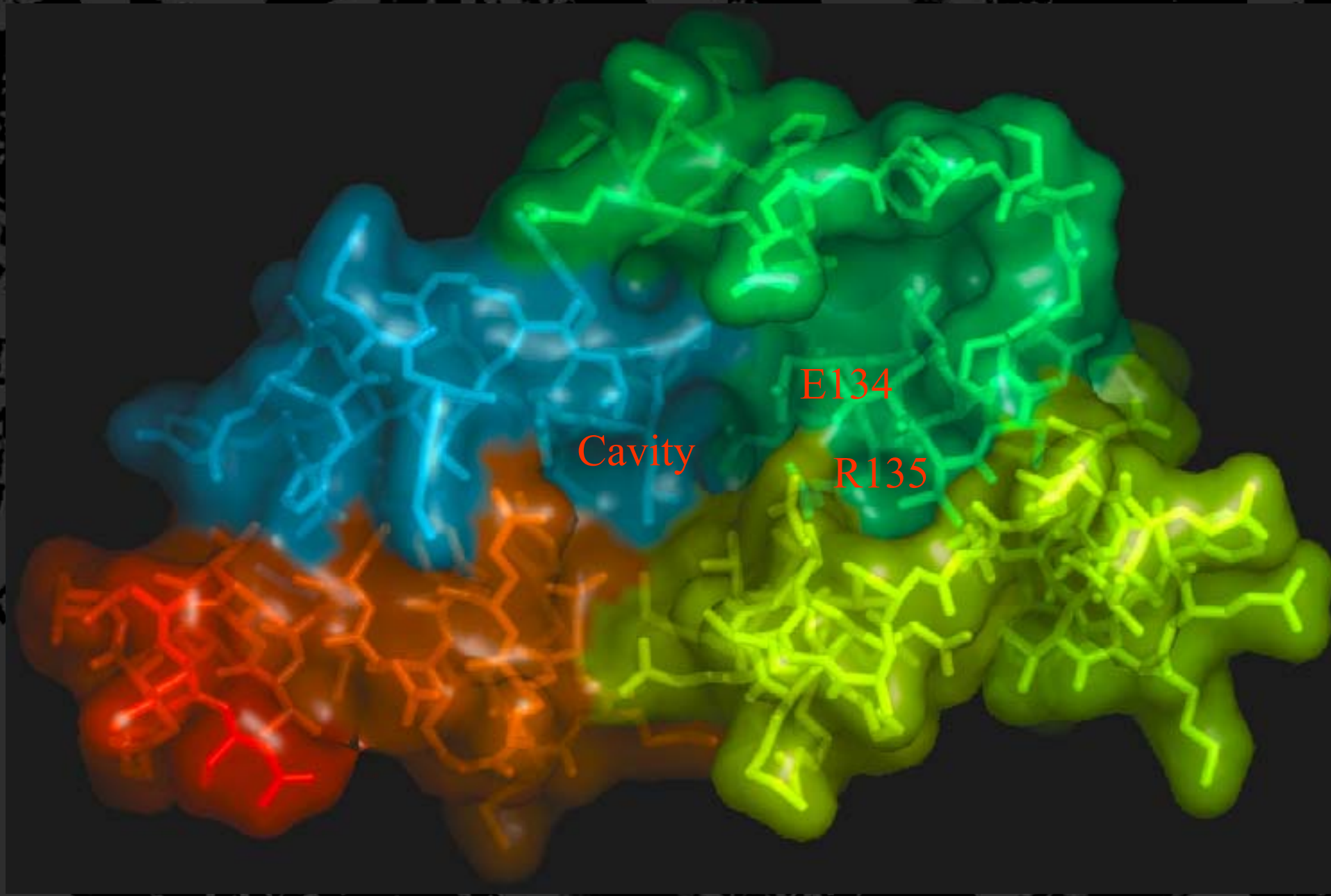
Rhodopsin lignad binding site:beta-ionon ring interactions



Transmission to NPXXY and E(D)RY



Cavity close to E134 and R135



Photolysis pathway of rhodopsin

Rhodopsin (498 nm)

↓ ps

Bathorhodopsin (542 nm)

⇌ ns

BSI (477 nm)

↓ ns

Lumirhodopsin (497 nm)

↓ µs

Metarhodopsin I (478 nm)

H^+ / H^+ ⇕ ms

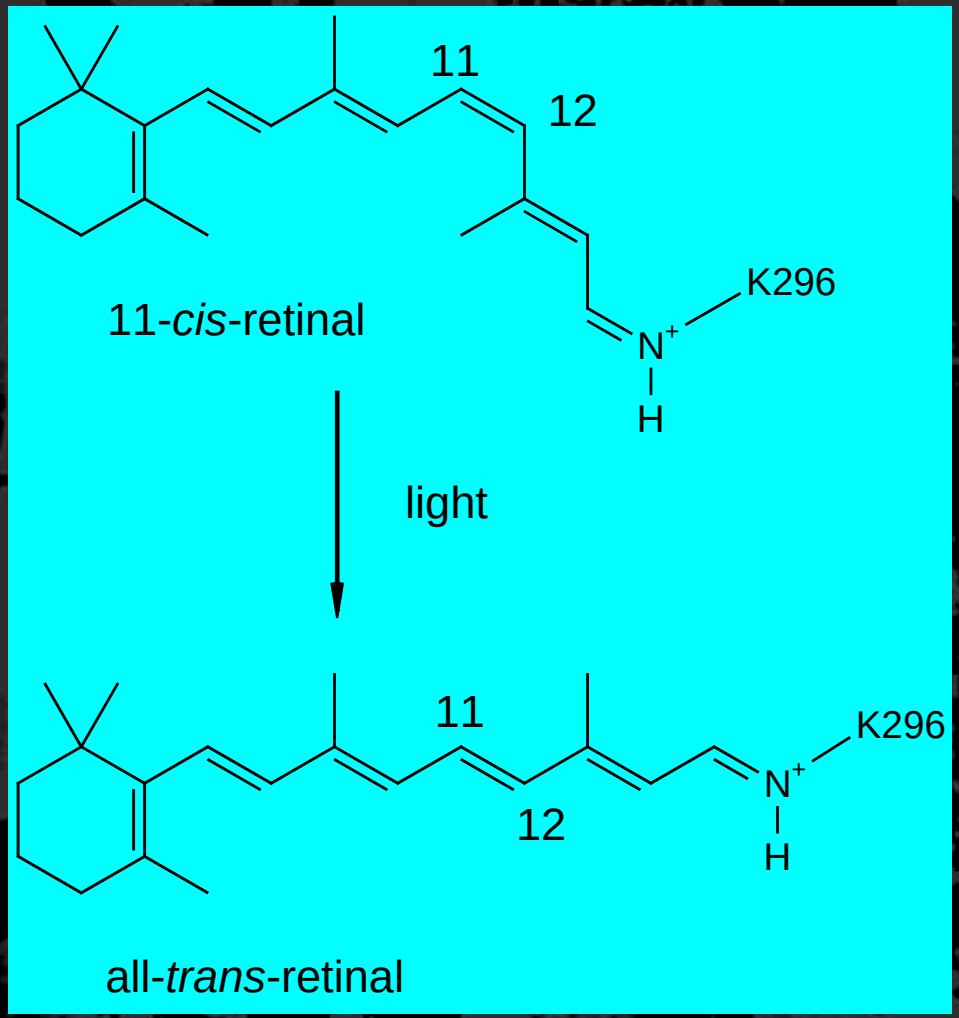
Metarhodopsin II (380 nm)

↓

Metarhodopsin III (465 nm)

↓

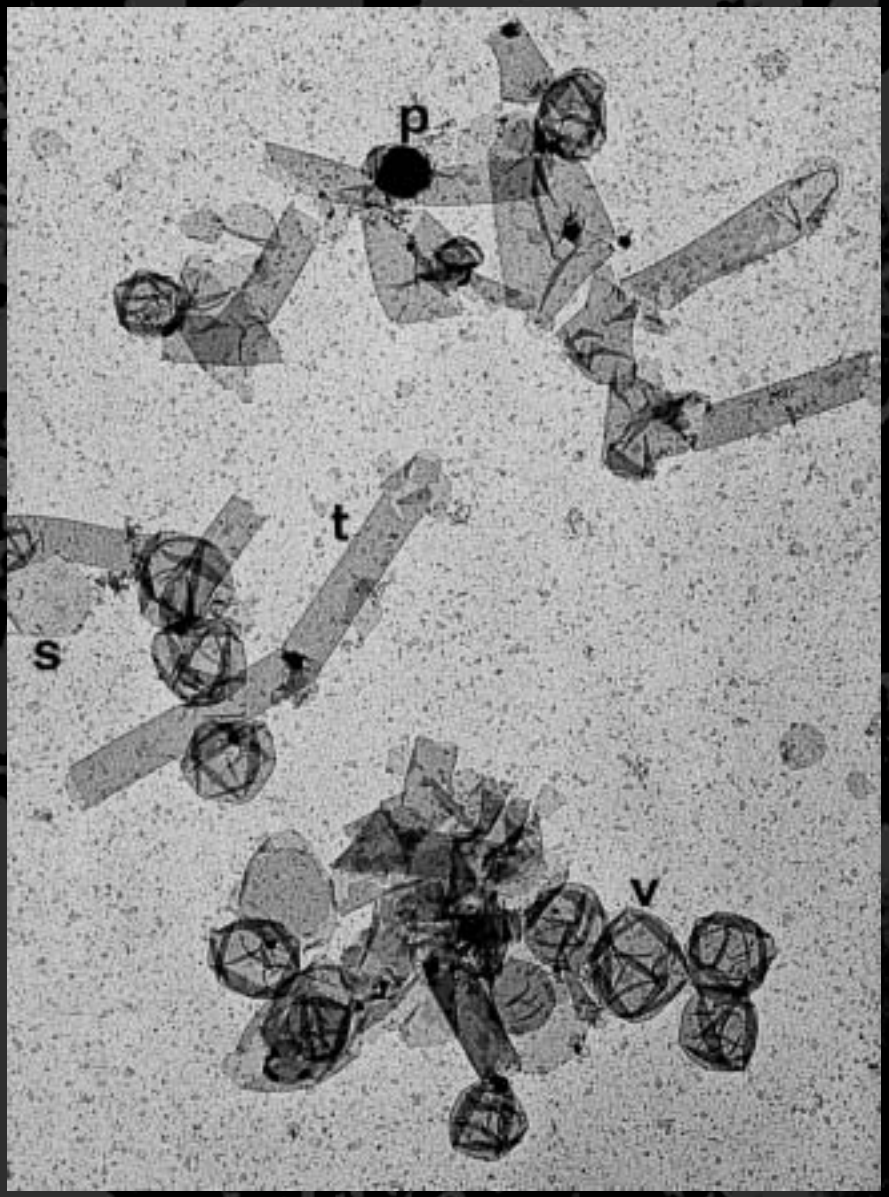
Opsin + free all-*trans*-Retinal (387 nm)

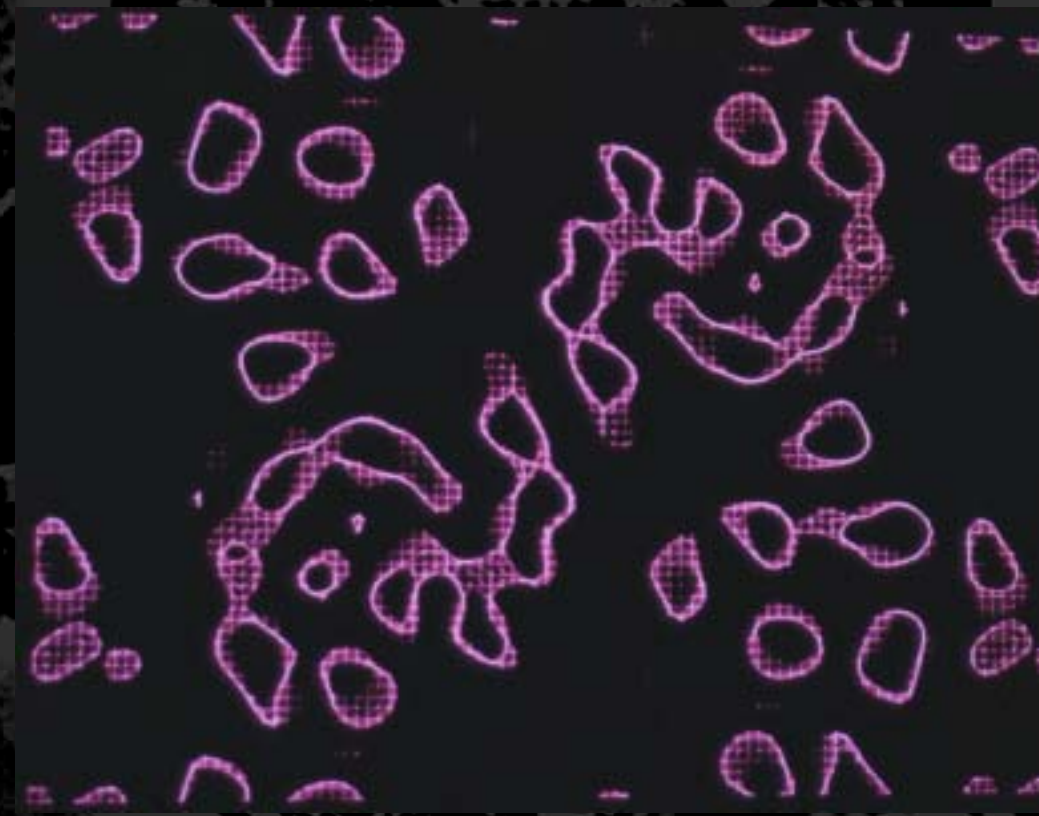


After light no x-ray diffraction!!!!

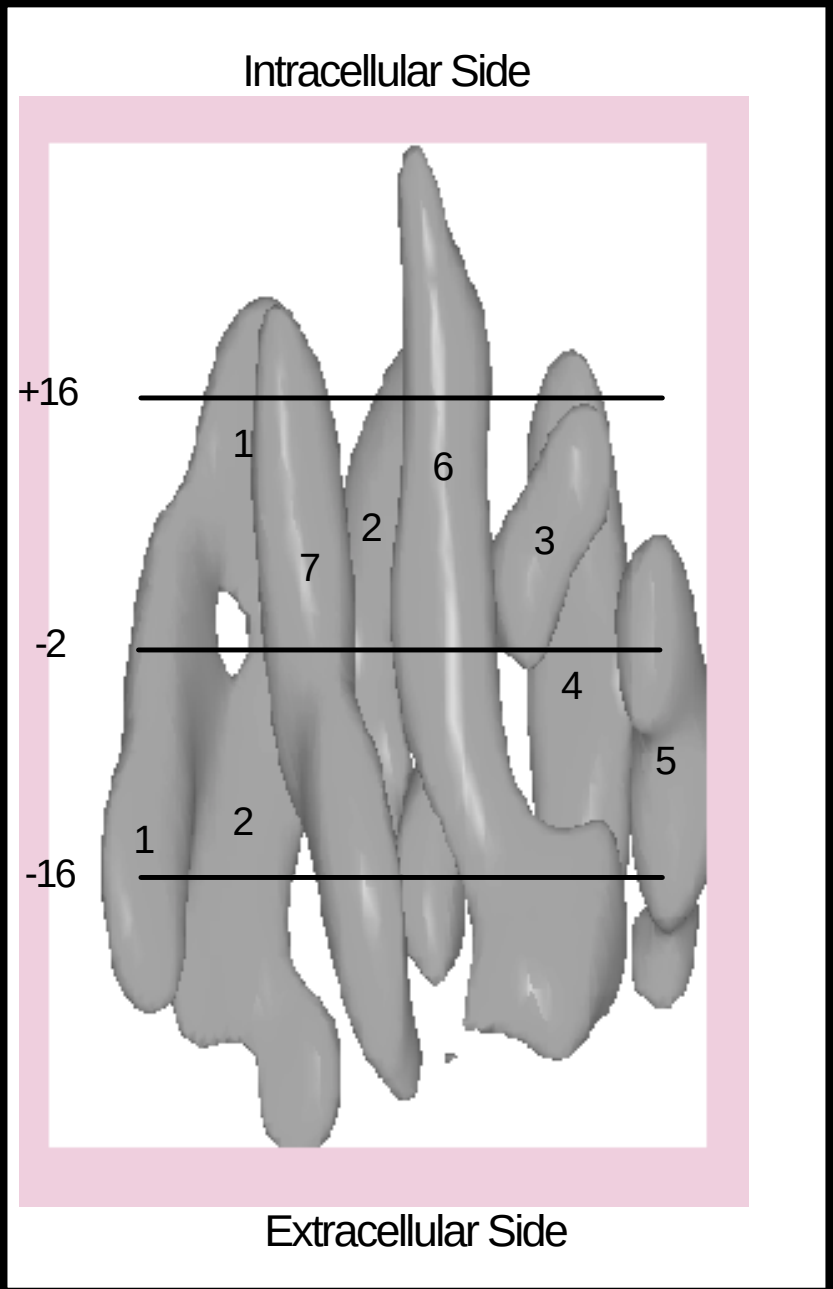
What next????

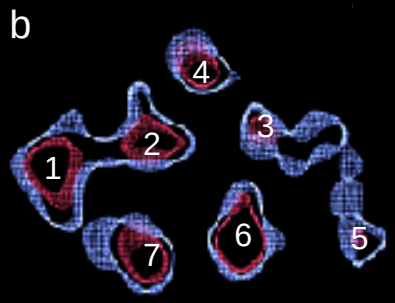
2D Crystal Bovine Rhodopsin p22121

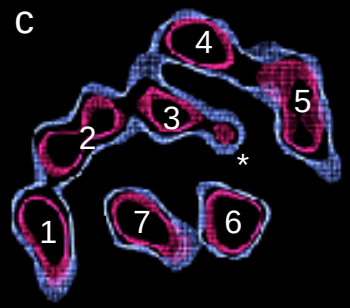


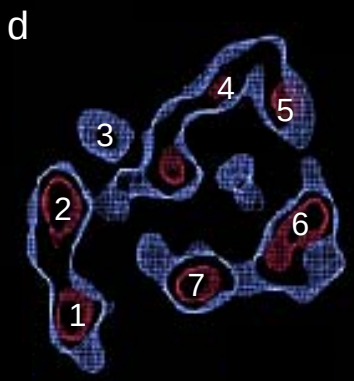


EM 3D Structure of Bovine Rhodopsin



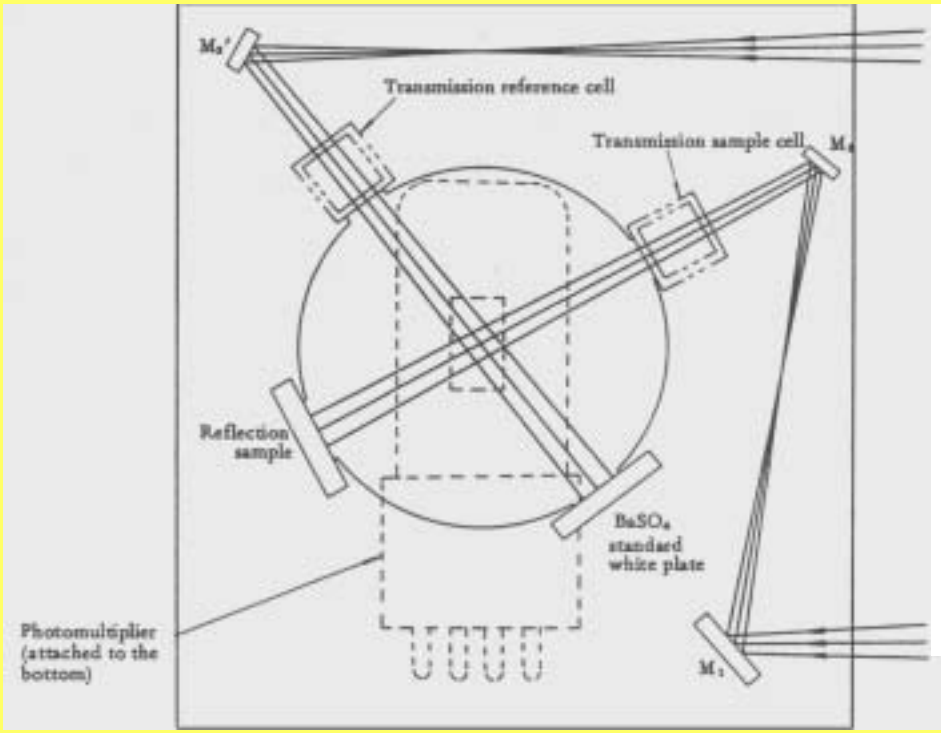


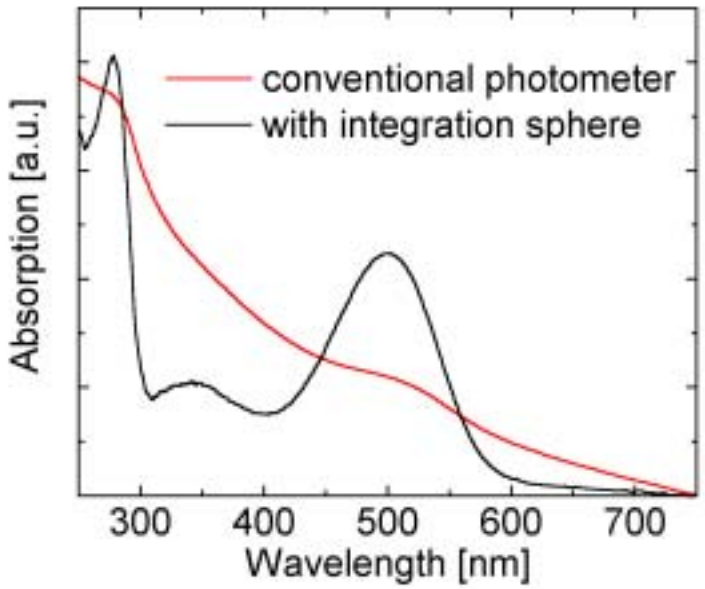


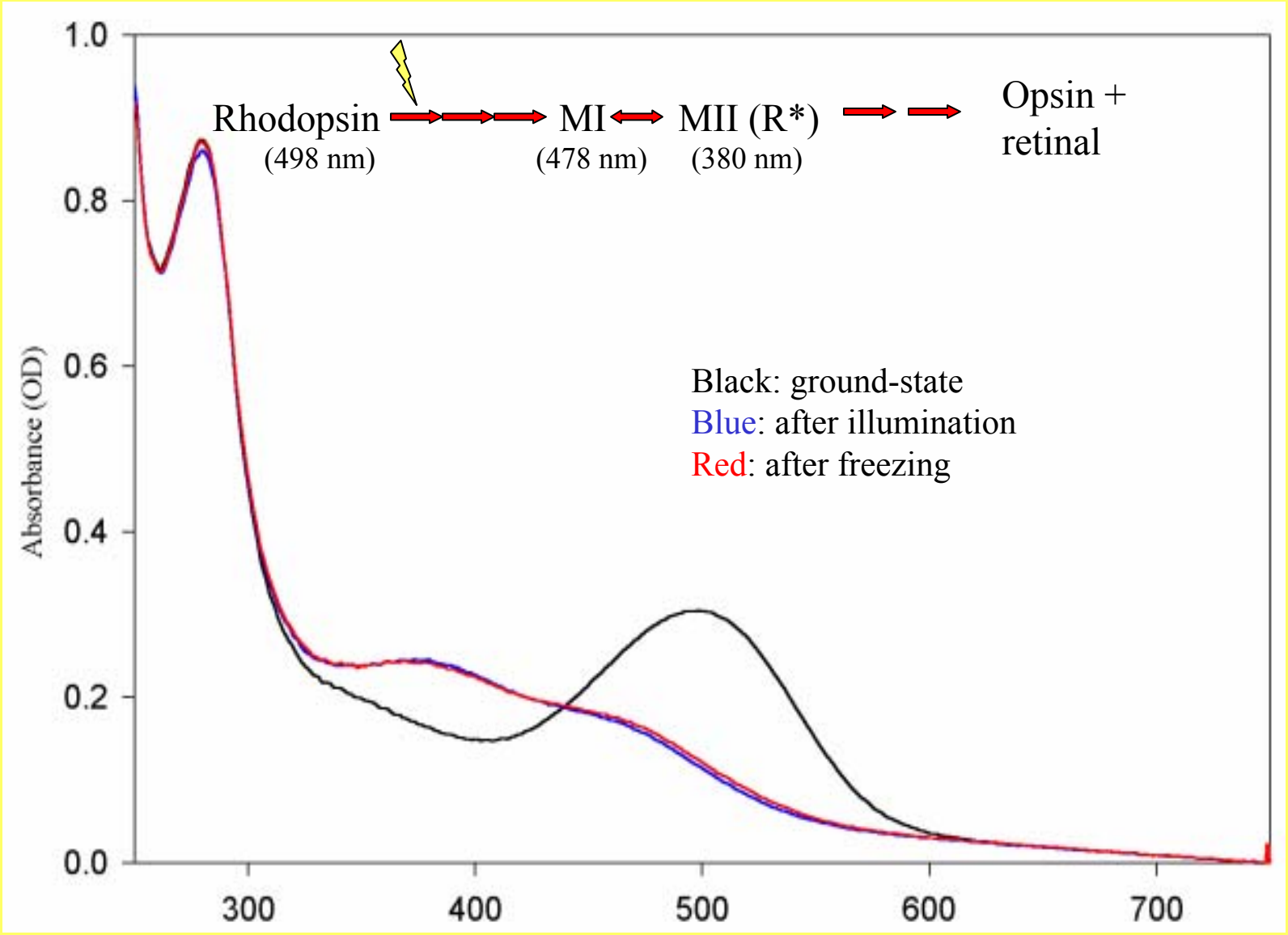


Photometer setup: - integration sphere

- temperature controlled cell holders

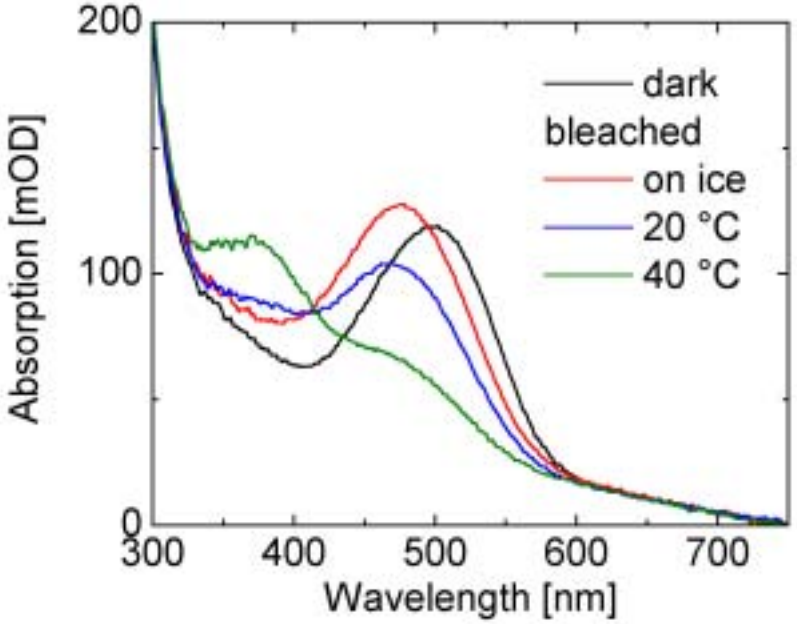




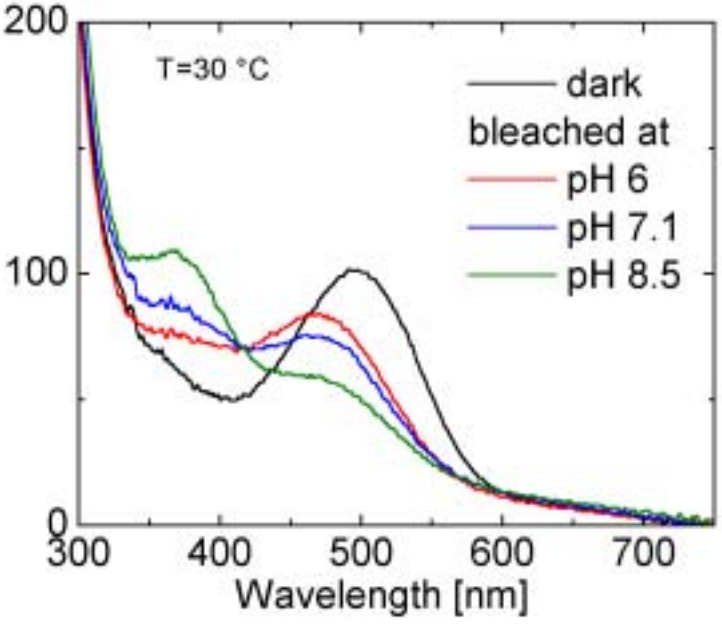

Rhodopsin
(498 nm)
MI
(478 nm)
MII (R*)
(380 nm)
Opsin +
retinal
Black: ground-state
Blue: after illumination
Red: after freezing
Absorbance (OD)
1.0
0.8
0.6
0.4
0.2
0.0
300
400
500
600
700

The MI/MII equilibrium in 2D crystals

A) temperature dependence:



B) pH dependence:



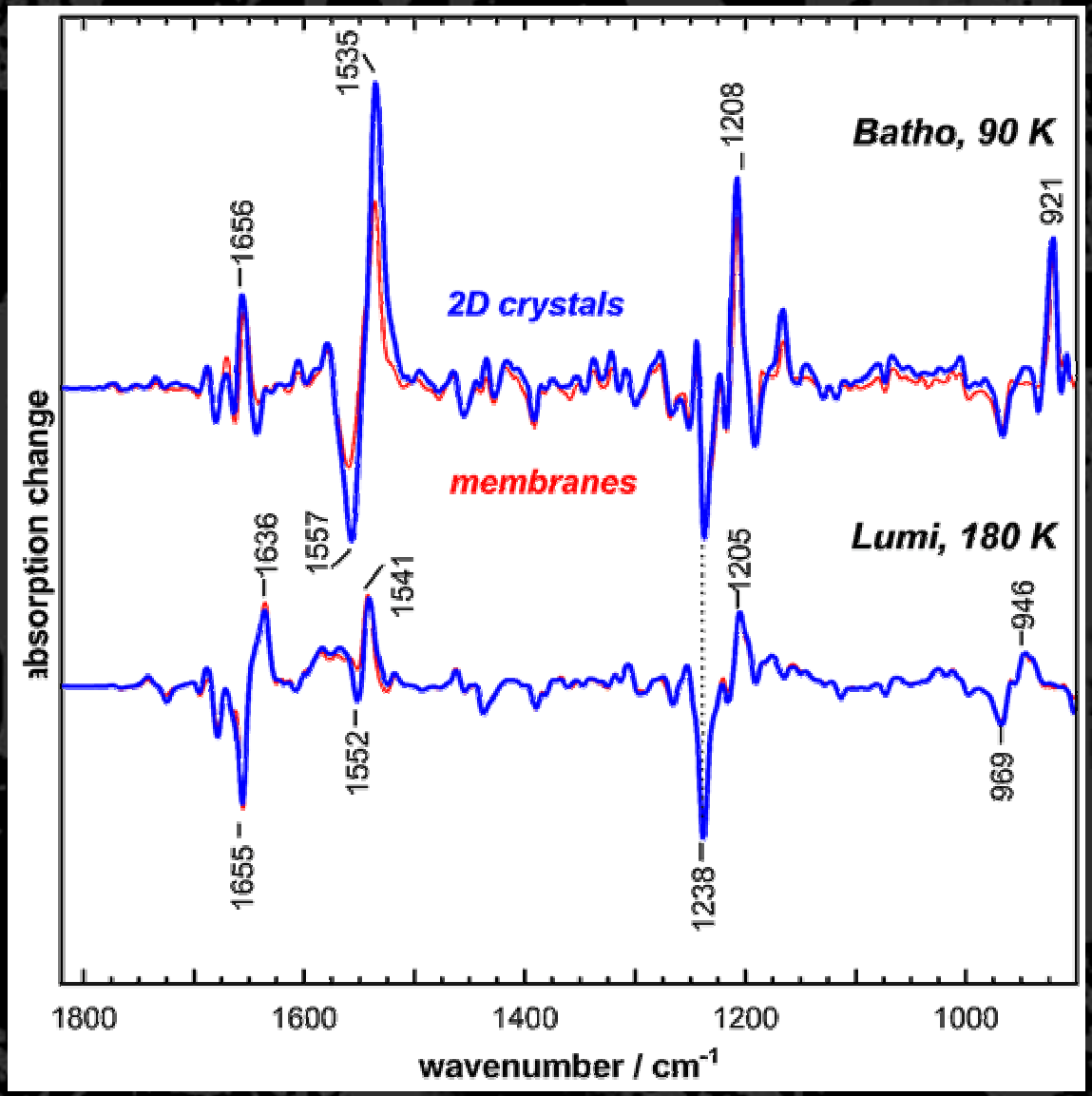
MI and MII like states in 2D crystals of rhodopsin???

MI

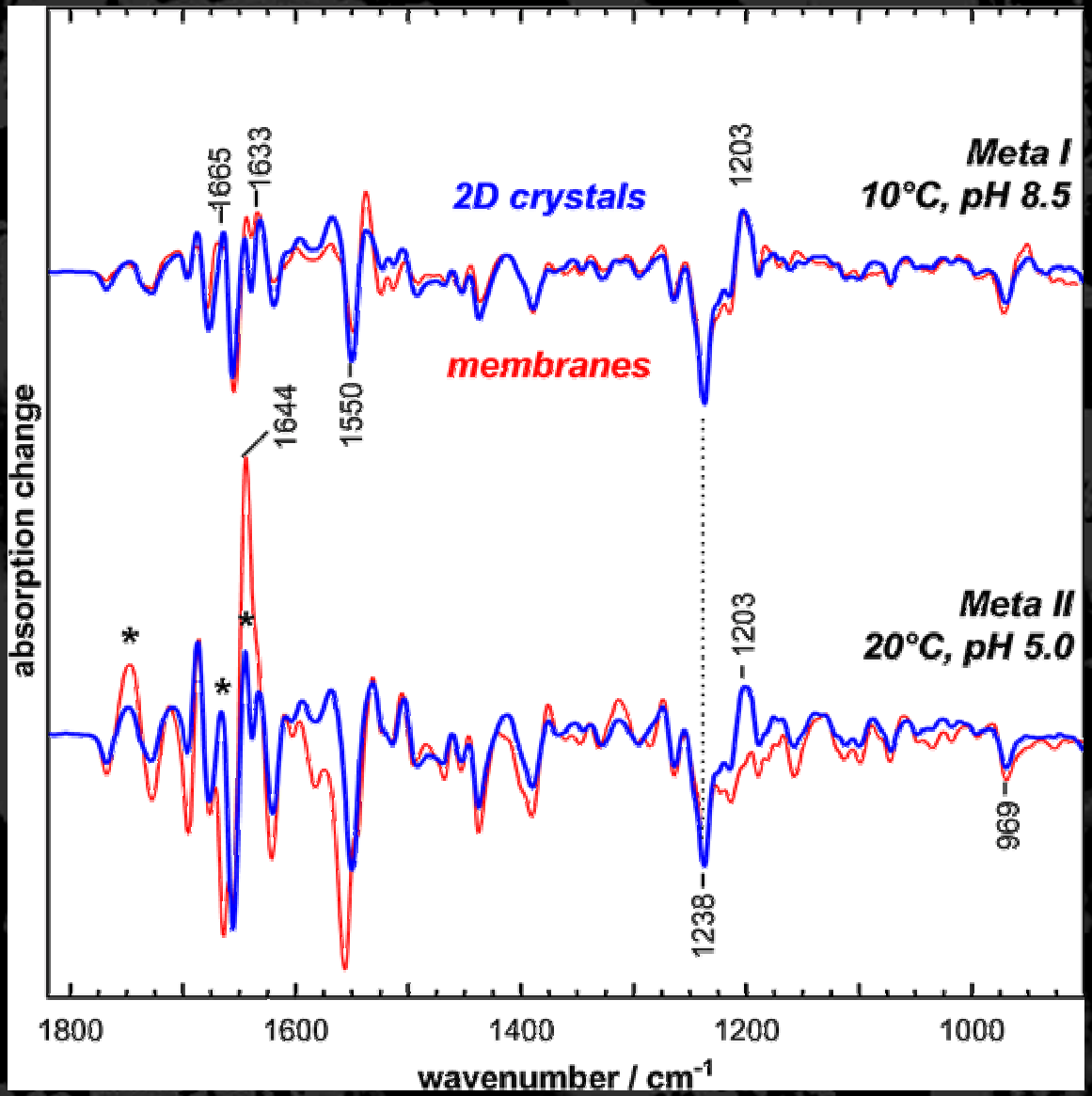
MI < = > MII

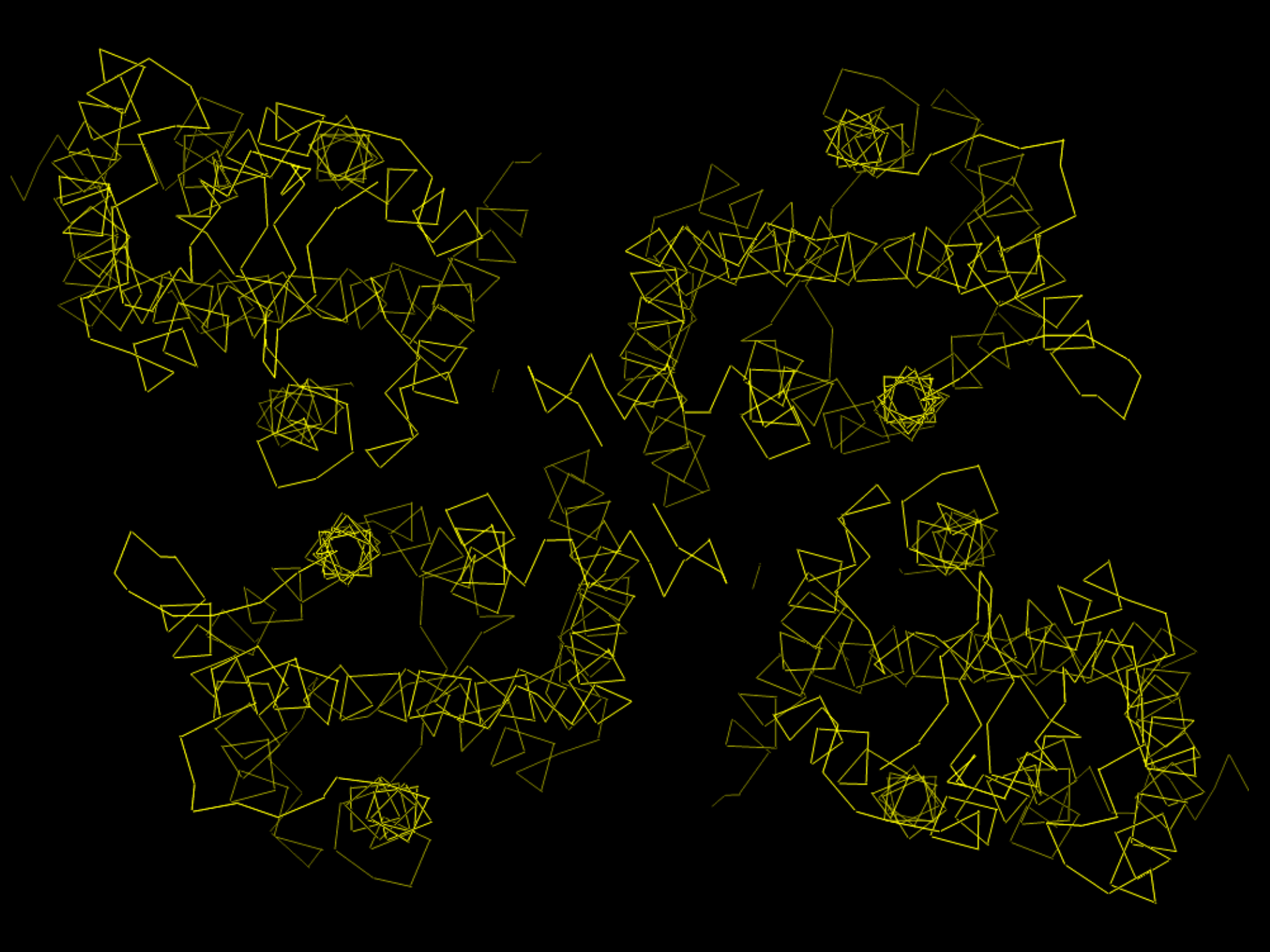
MII

The Low Temperature Photointermediates in 2D Rhodopsin Crystals



The Meta Photoproducts in 2DRhodopsin Crystals

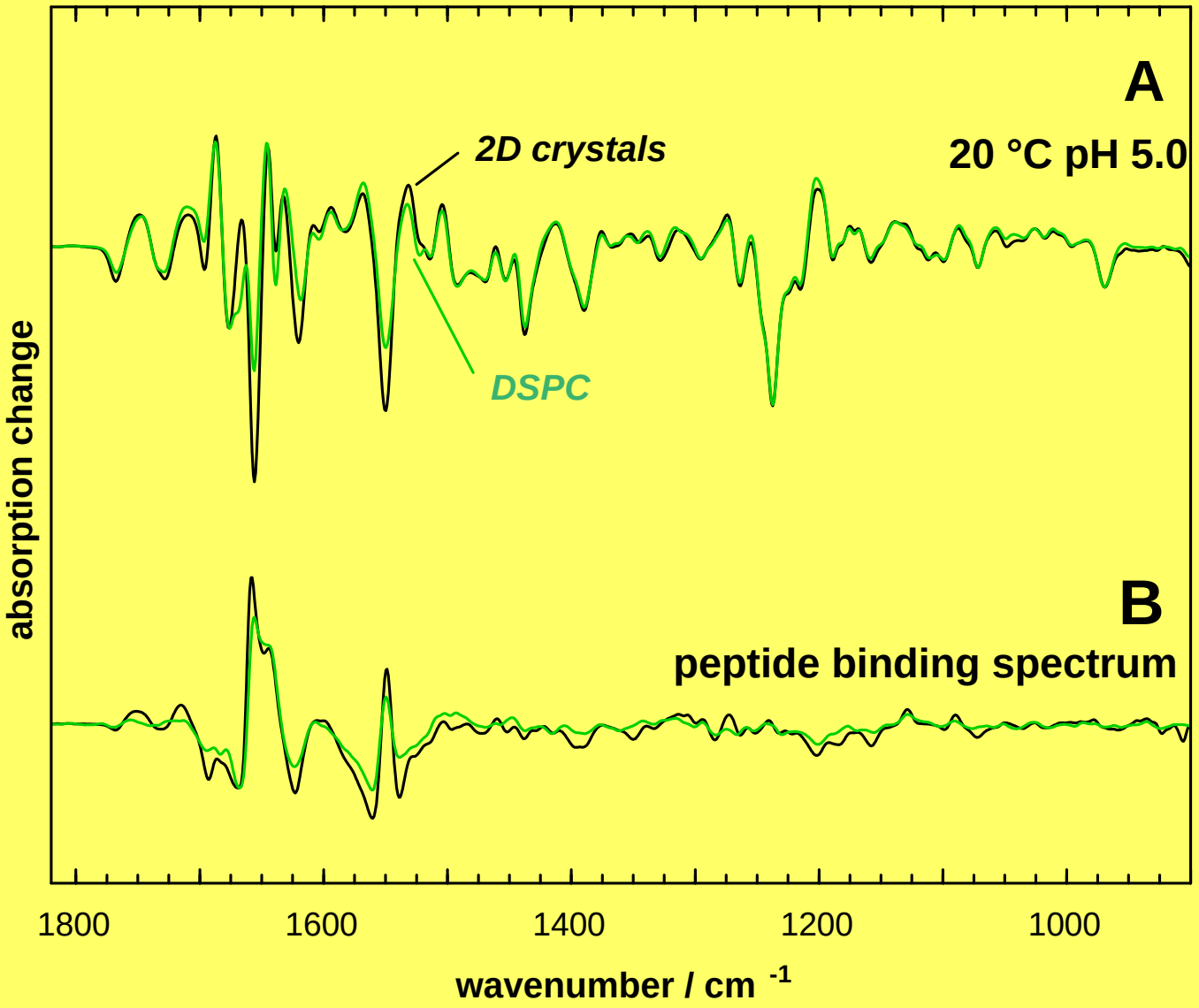




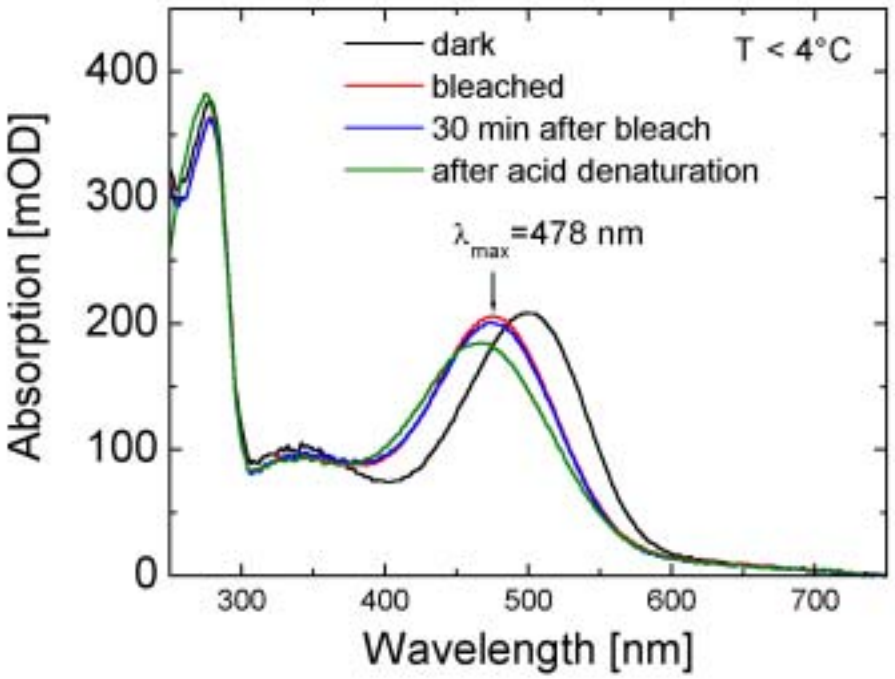
DSPC Mimicks the Crystalline Environment

DSPC

Di-stearoyl PC



Trapping the MI photointermediate in 2D crystals:



Equilibrate temperature (< 4°C)

↓

dark spectrum

↓

bleach (1 min, OG 515)

→ aliquot loaded on grid

↓

freezing

↓

control spectrum

↓

acid denaturation, spectrum

Sample preparation and data collection

Temperature-controlled humidity chamber

Blotter

EM grid

Liquid ethane (-185°C to -196°C)

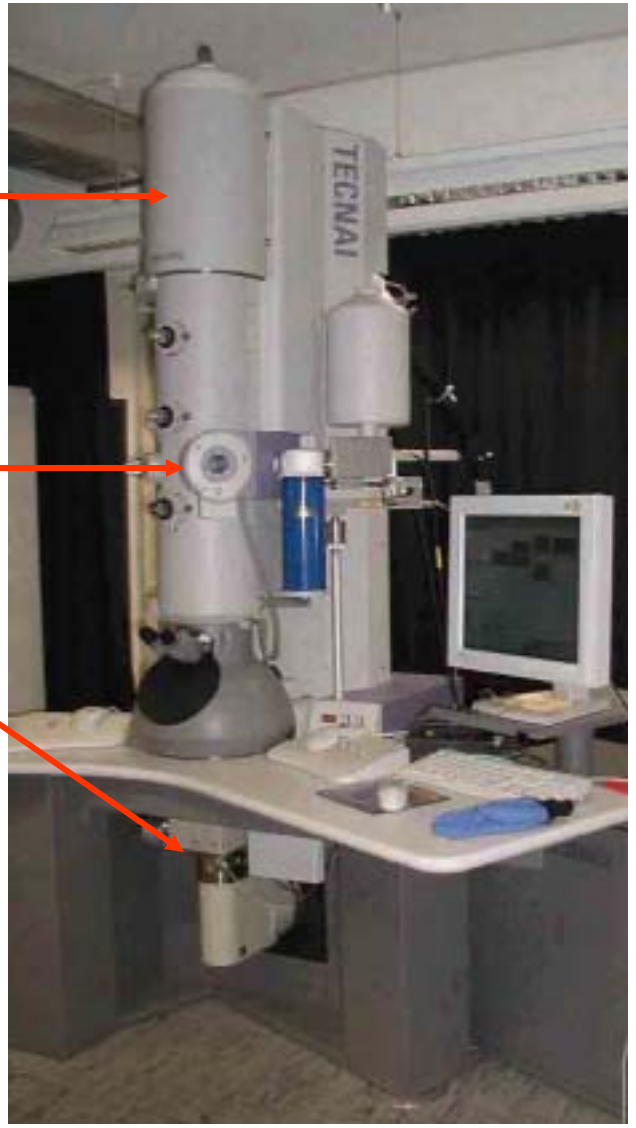
Liquid nitrogen

Tecnai F30

300 kV FEG

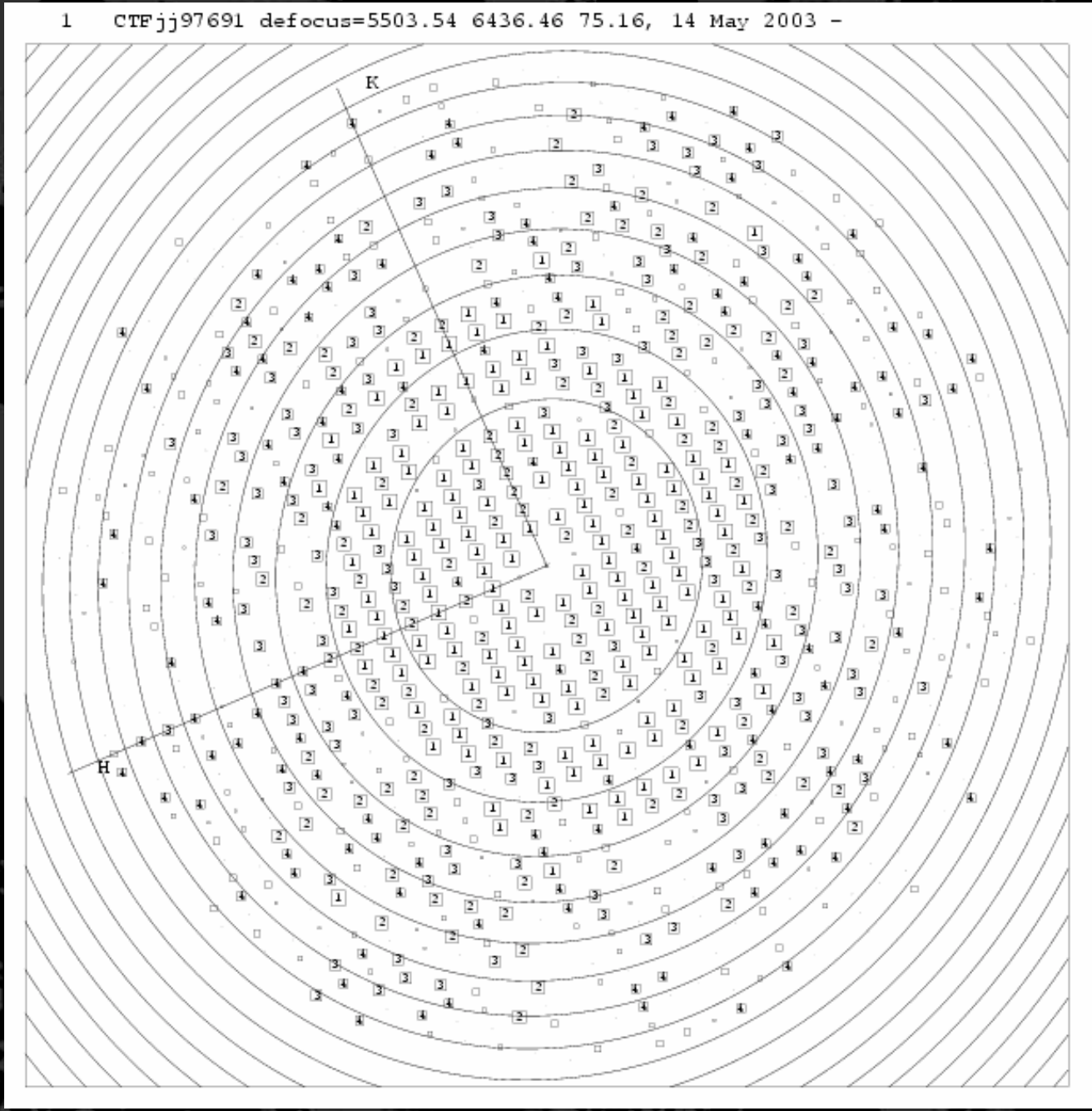
Goniometer

Camera (film/CCD)

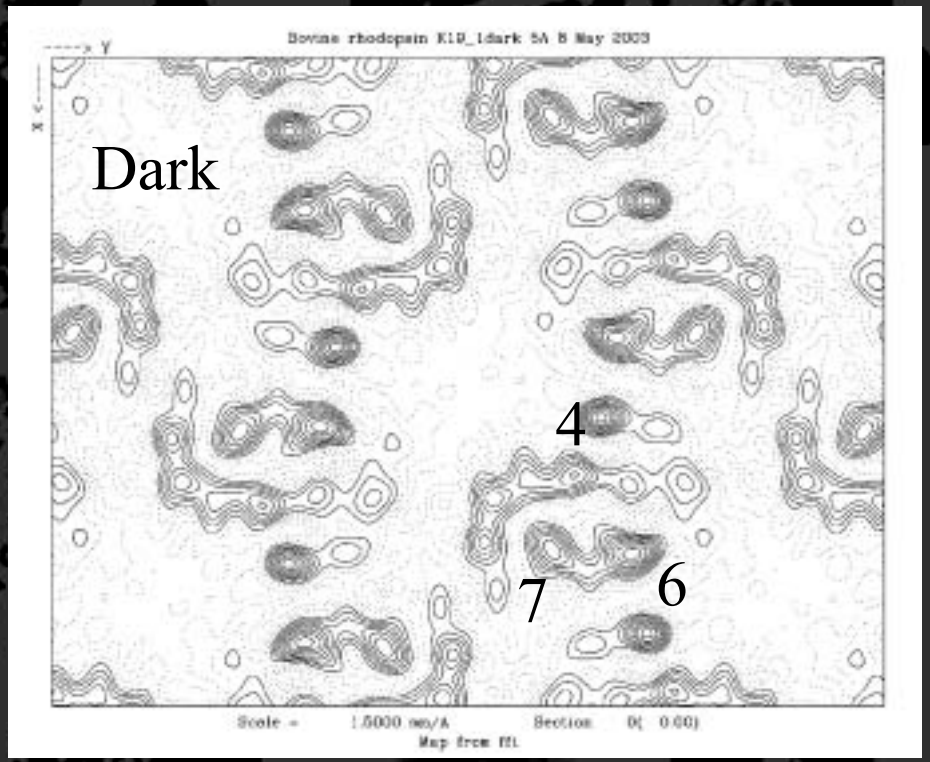


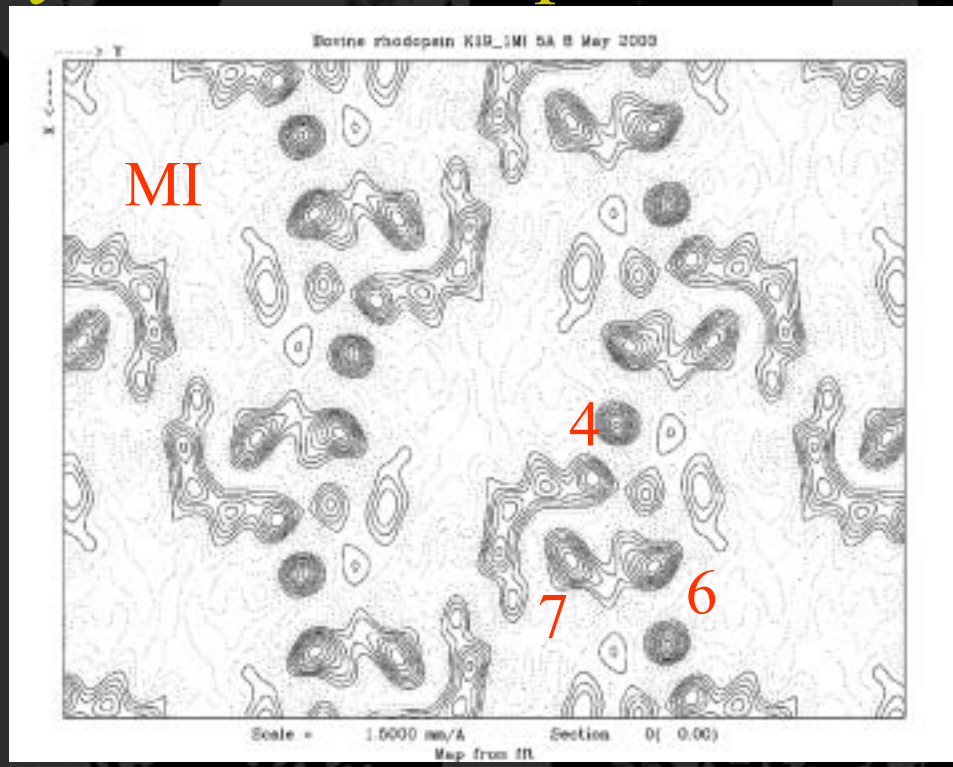
Calculated Fourier Transform of an Image of a MI Crystal

Good quality spots are present in the 5-3.5 Å range



MI states in 2D crystals of rhodopsin





MI Dark

Tomography

Reconstructed

3-D Structure

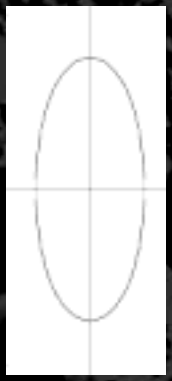
Electron crystallographic statistics

Table 1 Electron crystallographic data

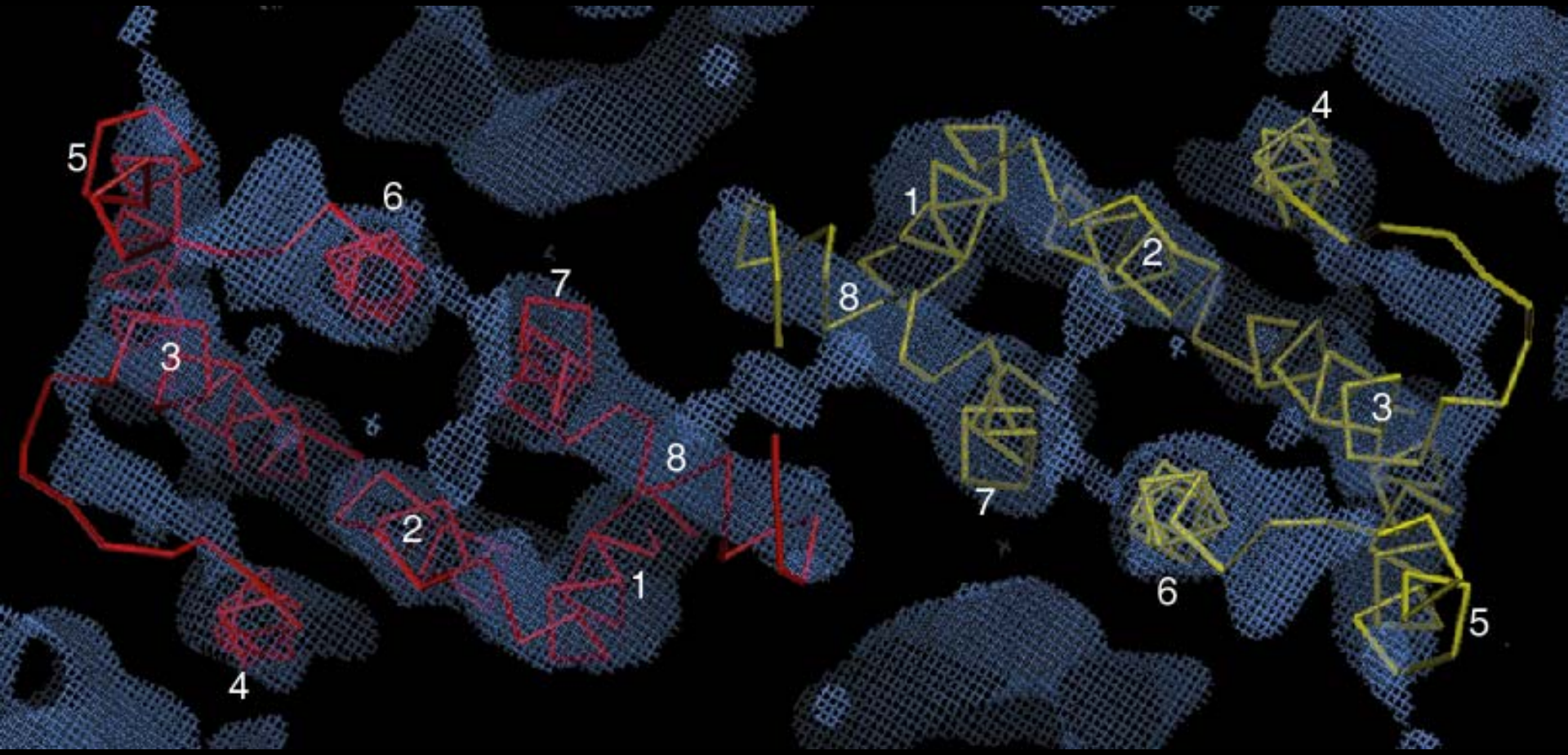
Plane group	$p22_12_1$
Cell dimensions	
a (Å)	58.8
b (Å)	83.7
c (Å)	200.0 (arbitrary)
$\alpha = \beta = \gamma$ (°)	90.0
Number of images[a]	87
Range of defocus (Å)	2700 - 14100
Effective resolution of 3D data set[b] (Å)	
In-plane	5.5
Perpendicular to the membrane	11.7
Average temperature factor (B_{xy})[c]	200 ± 95
Total number of observed amplitudes and phases	22099
Number of unique structure factors	2073
Completeness (%)	
0-45°	83.2
0-60°	68.2
0-90°	59.0
Overall weighted R-factor[d] (%)	34.9
Overall weighted phase residual[d] (°)	24.0

[a]Twenty-two at 0°, sixteen at 20°, twelve at 35°, twenty-six at 45° and eleven at 60°.
[b]As calculated from the point-spread function.
[c]Average temperature factor to scale image amplitudes against bacteriorhodopsin electron diffraction data, correcting for high-resolution image amplitude fall-off.
[d]From program LATLINEK.

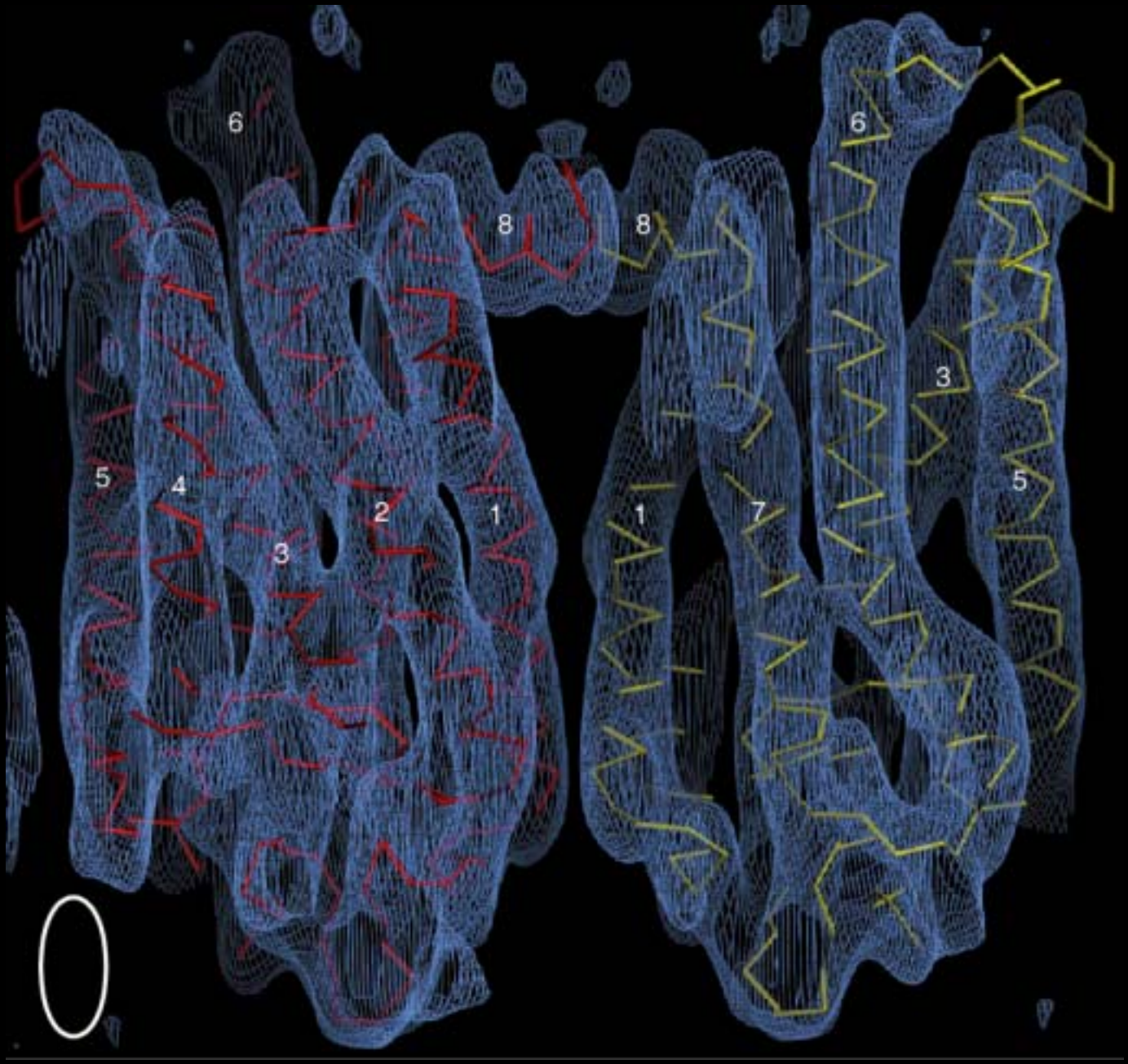
Point spread function



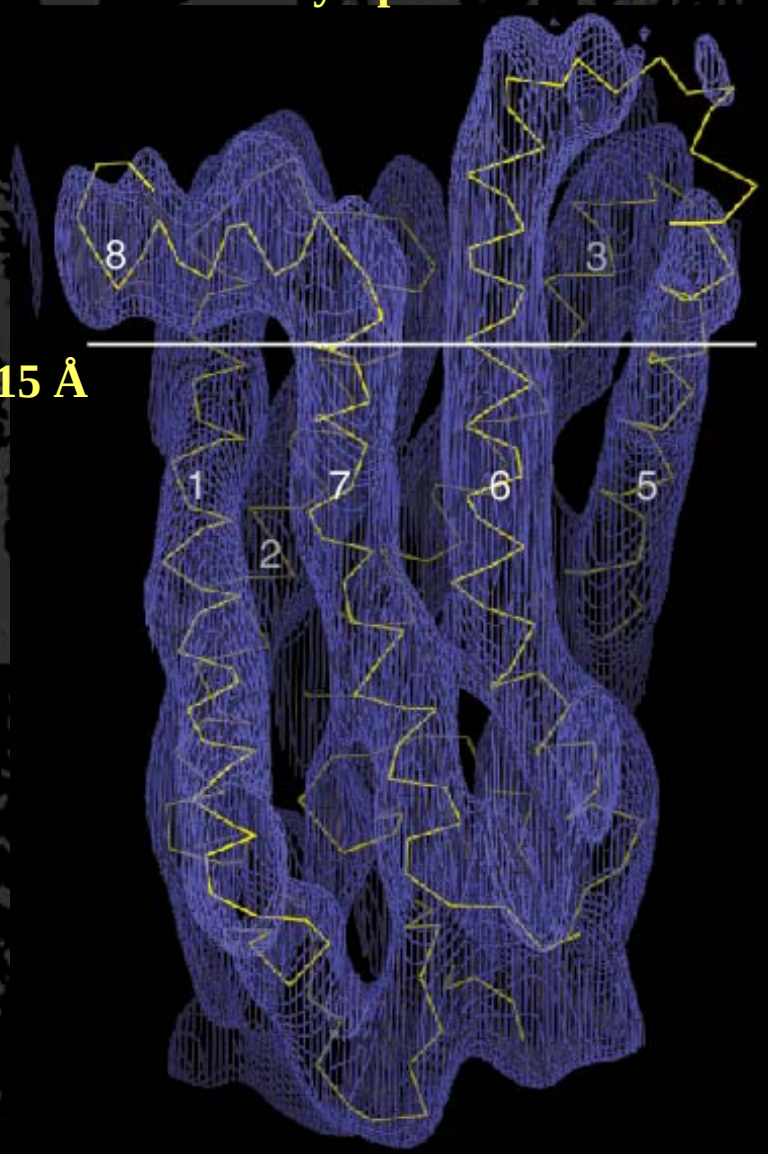
3D structure of the MI state

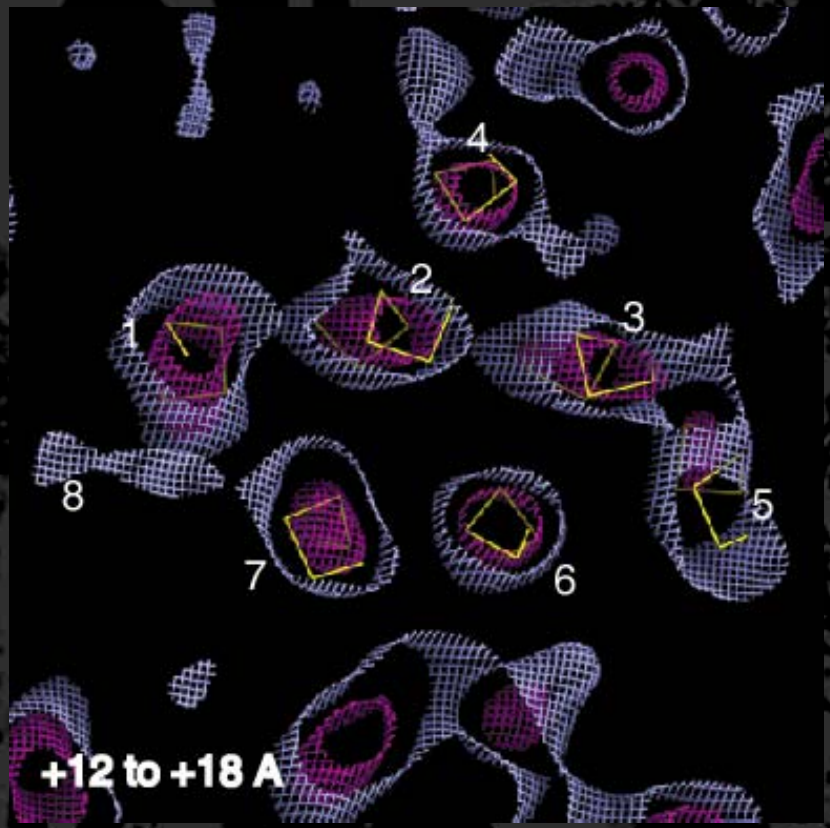


3D structure of the MI state



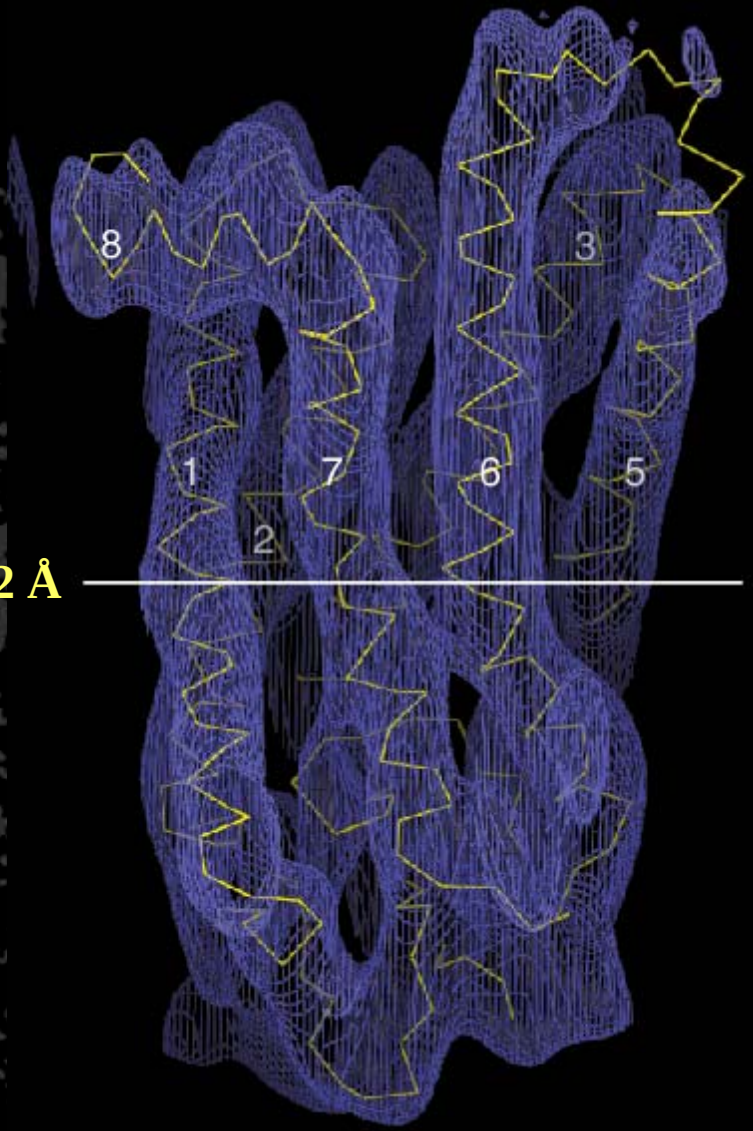
Cytoplasmic side



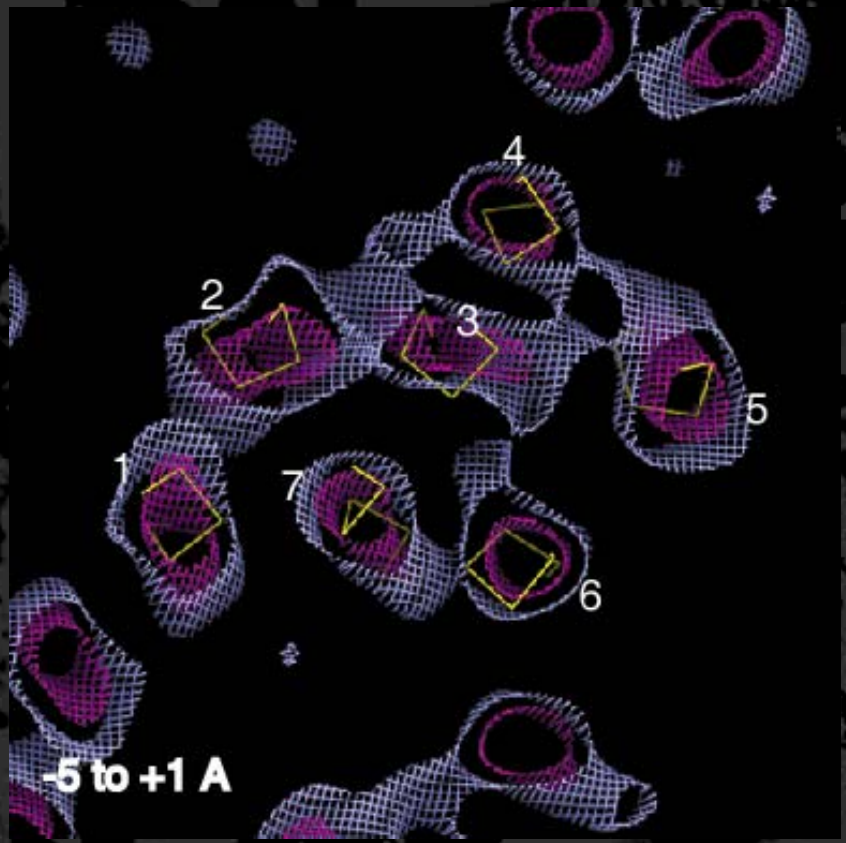


Extracellular side

Cytoplasmic side

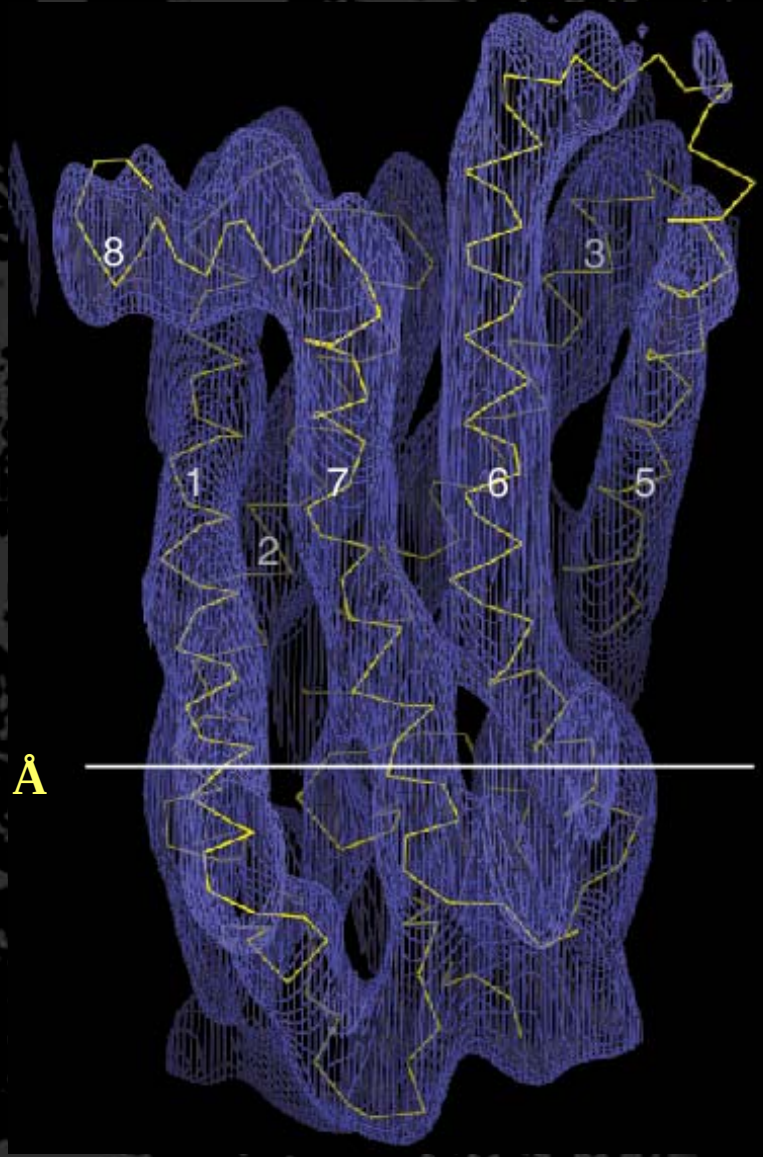


-2 Å



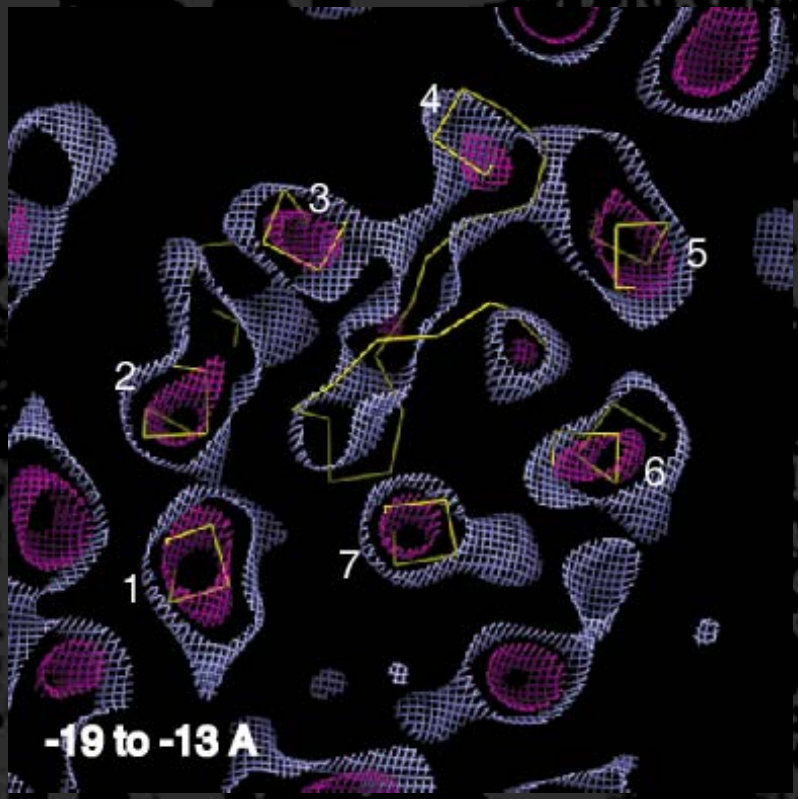
Extracellular side

Cytoplasmic side

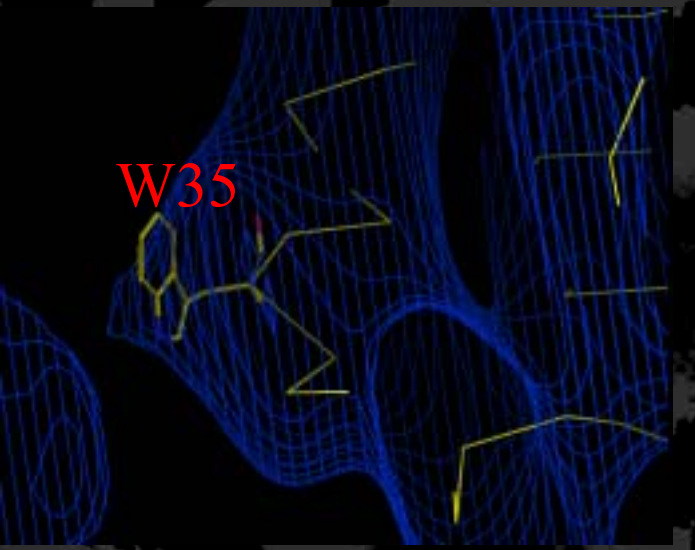


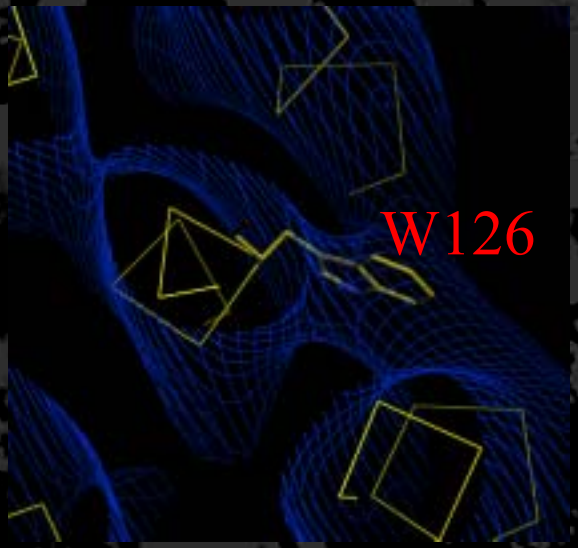
-16 Å

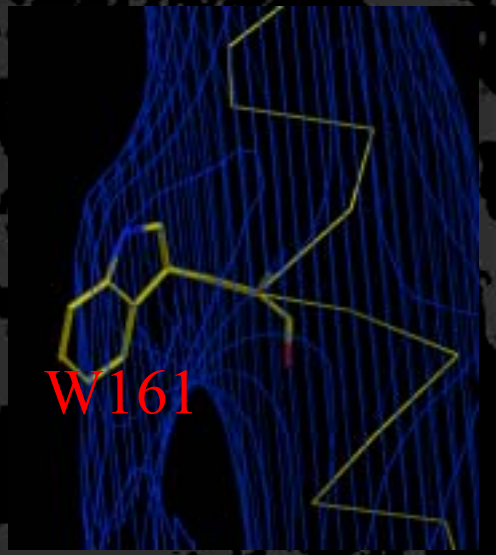
Extracellular side

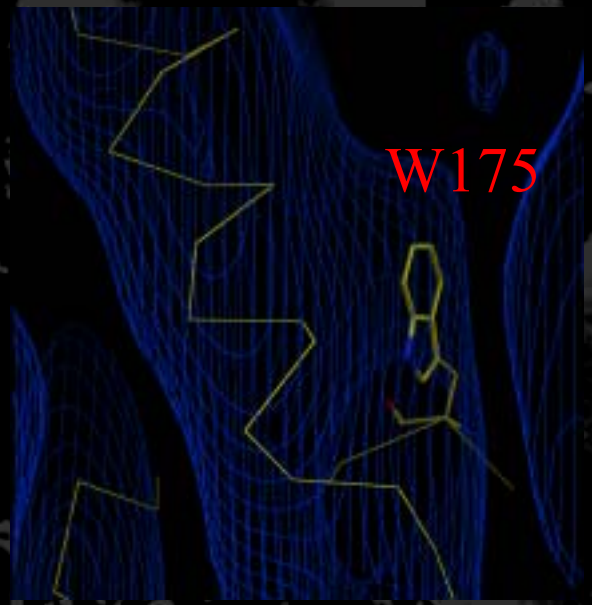


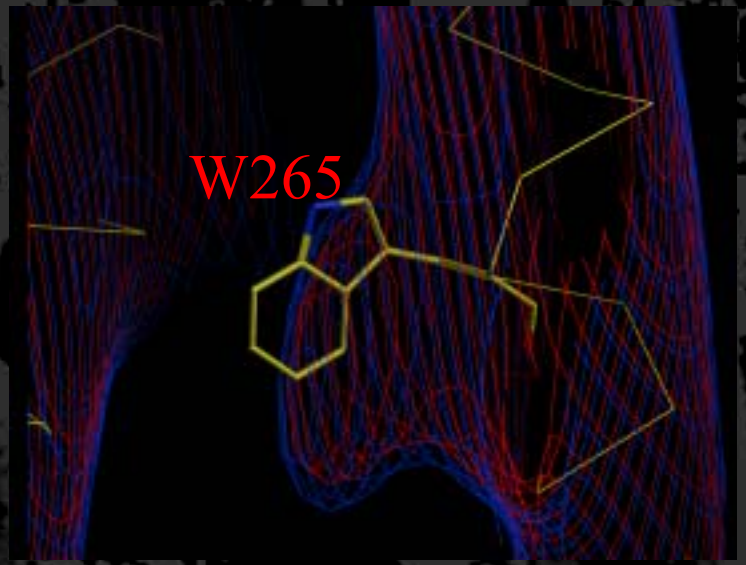
Density Features for all 5 Tryptophanes in Metarhodopsin I



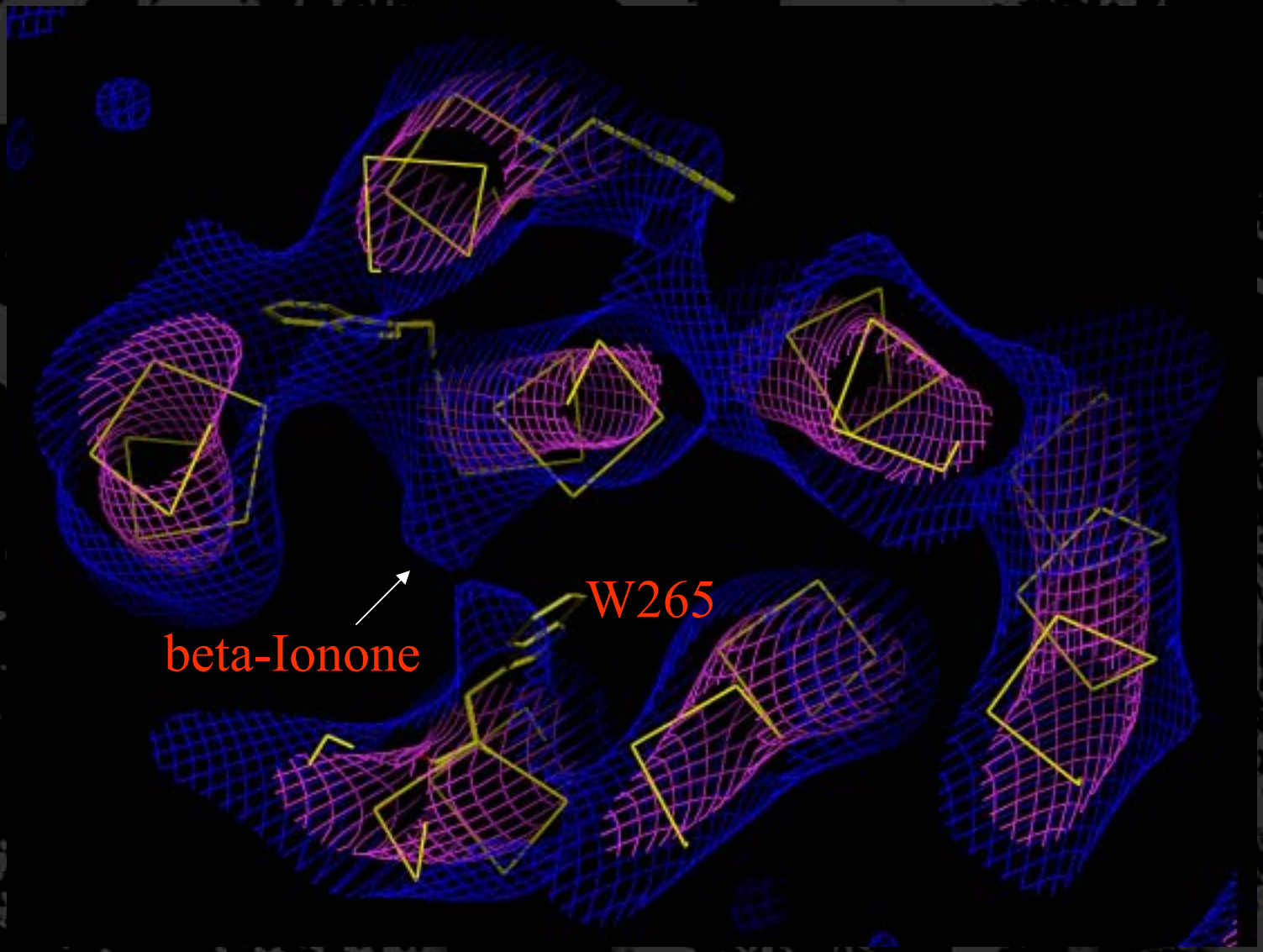








Density Feature for beta-Ionone Ring



Conclusions

- Rhodopsin photointermediates formed in 2D crystals reflect those formed in the native membrane up to metarhodopsin I.
- UV/Vis and infra-red spectra prove that the MI intermediate in the crystal is very similar to the MI intermediate in the disk membrane.
- Crystals of MI retain the ability to diffract and can be processed to yield a 3D density map.
- There are no large conformational changes in all eight helices in MI. Some deviations are observed close to the bend in helix 6.
- Features for all 5 tryptophane residues are visible, only W265 has shifted and rotated.
- A density feature close to helix 3 indicates the position of the beta-ionone ring, it is very close to the position in the dark map.

Structure of Rhodopsin: Metarhodopsin I in 2D Crystals of Rhodopsin

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